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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA, PA.

A CONVULSIVE SYNDROME IN YOUNG RATS ASSOCIATED WITH PYRIDOXINE DEFICIENCY

ESTHER PETERSON DANIEL, O. L. KLINE AND CHESTER D. TOLLE

Vitamin Division, Food and Drug Administration, Washington, D. C.

(Received for publication October 15, 1941)

The use of the U.S.P. method of assay for thiamine [1] as a satisfactory and accurate procedure is now well established. In studies designed to demonstrate that the U.S.P. diet is complete in all respects except for thiamine (Kline, Tolle and Nelson, '38) it was found that animals reared to maturity on the thiamine test diet supplemented with crystalline thiamine were capable of normal reproduction. These and subsequent studies, however, revealed the inability of such animals to maintain their young normally through the period of lactation. A syndrome occurring in the young during the suckling stage was characterized by frantic running about the cage, accompanied by loud cries and followed by convulsive seizures. This condition was not cured by administration of crystalline thiamine, yeast, or liver extract. It was demonstrated, however, that the convulsive syndrome in the suckling young bore no relationship to the nutritional state of animals being used for thiamine assay and had no influence upon results of the assay method conducted in accordance with the specified U.S.P. procedure. With animals employed for this assay the administration of relatively large doses of riboflavin, pyridoxine, nicotinic acid, pantothenic acid or choline, in addition to the usual curative dose of thiamine standard, was found to have

[1] The Second Supplement to the Pharmacopoeia of the United States of America, Eleventh Decennial Revision, p. 129.

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no effect upon the length of curative response to vitamin B_1 (Kline, Hall and Morgan, '41). Only during the period of lactation was there any evidence of deficiency. Further study both of the deficiency syndrome in the young and its cure is the subject of this presentation.

EXPERIMENTAL

These studies involved the observation of a number of rats throughout the period of reproduction and lactation, and of litters of young produced under several experimental conditions. The females used were from two sources: stock animals changed to the experimental diets during the period of pregnancy, and animals reared on the experimental diets from weaning through maturity and reproduction. The animals were kept on raised screens in the usual manner except during a short period preceding parturition and the 15 days following. During this time they were caged on screens resting directly on sawdust. Diet and water were available ad libitum. Records were kept of birth of litters, of weights of mothers and young taken at regular intervals, and of observations of condition of the animals throughout the suckling stage. The composition of the diets fed is given in table 1 and in table 2 is indicated the number of animals studied on each of these diets.

Twenty adult females were placed on diet 22E, a ration which had been demonstrated to be adequate for normal growth and reproduction. This is a ration used in the laboratory for thiamine assay which has been supplemented with 200 μg. each of crystalline thiamine and riboflavin per 100 gm. of diet. Thirteen of the twenty females studied had been reared from weaning to maturity on this diet, two were stock animals changed from the stock diet during pregnancy and five were second-generation animals reared on diet 22E.

In an attempt to influence the symptoms which developed in the young born to these females a number of supplements were administered orally to both the young and mother as indicated in table 2. In several litters stock colony young of the same

age were substituted for a part of the litter of mothers on diet 22E and the experimental young in turn were given to the stock females retained on the stock diet. Treatment of the mother included administration of alpha-tocopherol, administration of pyridoxine, both by oral feeding and supplementation of the diet, and modification of the diet to omit the autoclaved peanuts. This modified diet is referred to as diet 23E.

TABLE 1

Composition of diets.

INGREDIENTS IN DIET	DIET 22E	DIET 23E	DIET 64B	DIET 65	DIET 69
	Grams per 100 gm.				
Casein [1]	18	18	18	18	18
Salts U.S.P. no. 1	4	4	4	4	4
Cod liver oil	2	2	1	1	
Sucrose	60	70	72	72	78
Autoclaved yeast	5	5			
Autoclaved peanuts	10				
Liver extract (irrad.)			1		
Liver extract (sulphite-treated)	1	1			
Corn oil			5	5	
	Micrograms per 100 gm.				
Thiamine	200	200	200	200	200
Riboflavin	200	200	300	300	300
Calcium pantothenate			500	500	500
	International Units per 100 gm.				
Vitamin D (irrad. ergosterol)					300
Vitamin A concentrate					300

[1] The casein was either acid reprecipitated or sulphite treated.

Eight adult females were placed during pregnancy on diet 64B, a more highly synthetic mixture containing purified sources of protein and carbohydrate and including 1% liver extract of the type ordinarily used as a source of the pernicious anemia factor. This ration was deficient in pyridoxine. The animals placed on this diet were a stock female trans-

ferred from the stock ration during pregnancy and seven animals which had been reared to maturity on diet 64B supplemented with pyridoxine.

TABLE 2

Summary of animals, diets and supplements.

TOTAL NUMBER OF ADULT FEMALES	DIET	TOTAL NUMBER OF LITTERS	TOTAL NUMBER OF YOUNG	MODIFICATIONS OF DIET		RESULTS
				Female	Young	
20	22E	37	360	None	None	Failure of young
				Alpha-tocopherol	None	Failure of young
				Peanuts omitted	None	Failure of young
				Pyridoxine	None	Normal young
				None	Alpha-tocopherol	Failure of young
				None	Yeast	Failure of young
				None	Ca pantothenate	Failure of young
				None	Corn oil	Failure of young
				None	Suckled by stock ♀	Normal young
				None	Pyridoxine	Normal young
8	64B	15	131	None	None	Failure of young
				None	Corn oil	Failure of young
				Pyridoxine	None	Normal young
2	65	2	20	None	None	Failure of young
2	69	3	39	None	None	Failure of young
				Pyridoxine	None	Normal young

Two adult stock females were transferred during pregnancy to each of diets 65 and 69, both of which were similar to diet 64B except for the omission of liver extract. Each ration was deficient in pyridoxine.[2] In diet 69 a concentrate of vitamins A and D was used in place of the cod liver oil and the corn oil was omitted.

RESULTS

A general summary of the results of these experiments is shown in table 2. Table 3 gives more detailed information on typical animals showing results which are representative of those obtained throughout the study.

[2] The terms vitamin B_6 and pyridoxine are synonymous and are used interchangeably in this presentation.

On each of the diets 22E, 64B, 65 and 69 normal reproduction was obtained but failure of the young was noted during the lactation period. Characteristic symptoms developed in the young usually between the eleventh and eighteenth days. These animals suddenly appeared emaciated, showed tremor, uttered sharp cries apparently due to pain, exhibited a series of running fits accompanied or followed by convulsions and in most cases by death on the same or following day. Even the young from stock females placed on these experimental diets a day or two before parturition exhibited such symptoms. The development of the deficiency was therefore not dependent upon maintenance of the female on any one of these diets for an extended time before birth of the young.

Normal stock colony young suckled by mothers on diet 22E from the first day after birth developed these same deficiency symptoms. In these cases, however, the onset of the symptoms was delayed and a small proportion of the animals survived. On the other hand, it was found that young from diet 22E females when placed with normal, lactating stock females receiving the stock ration developed normally in every respect.

That lack of vitamin E was not the cause of the symptoms was demonstrated by feeding synthetic *dl*-alpha-tocopherol at different times to both the female and the young, after which the characteristic condition still occurred. Neither was there any alleviation of the symptoms by omitting the autoclaved peanuts from diet 22E. This was done to eliminate any possible destruction of vitamin E that might have occurred as a result of rancidity in this material. Calcium pantothenate in large doses administered orally to the young did not prevent the occurrence of the symptoms. The effect of yeast fed after the onset of the condition was questionable. Because of the rapid development of the symptoms it is possible that the yeast was not administered sufficiently early to allow effective absorption from the alimentary tract. Corn oil, given to the young, did not appear to have any marked effect except to prolong slightly the survival period. The symptoms were rapidly cured or prevented when pyridoxine was administered orally

TABLE 3

Records of typical animals.

DESCRIPTION OF FEMALE		DIET OF FEMALE	LITTER NUMBER	NUMBER OF YOUNG BORN[1]	PORTION OF THE LITTER RECEIVING SUPPLEMENT	
					Supplement	No. of young
Reared to maturity on diet 22E	1	22E	1	8		
		22E	2	8	Suckled by stock ♀ on stock diet	2
		Stock	3	9		
		22E + B_s daily	4	4		
	2	22E	1	11	Alpha-tocopherol on 13th day	2
		23E + alpha tocopherol	2	5		
		22E	3	9	Suckled by stock ♀ on stock diet	4
					Yeast 16th day (after tremor)	1
					B_s (after convulsions)	1
		22E + 40 μg. B_s daily	4	11		
	3	23E + alpha tocopherol	2	7		
		22E	3	6	Suckled by stock ♀ on stock diet	3
					B_s 15th day (after convulsions)	1
					Yeast 16th day (after convulsion	2
	4	22E	1	9	Alpha-tocopherol on 9th day	4
		22E	2	11	Ca pantothenate 13th and 15th d	4
	5	22E	2	8	Corn oil 13th day (after convulsions)	4
	6	22E	1	9	B_s (10 μg. daily 3rd–6th day then 20 μg. daily to weaning)	8
	7[2]	22E	1	5		
Pregnant stock ♀	8	22E	1	10	B_s (80 on 18th and 22nd day—after convulsions)	2
Reared to maturity on diet 64B + vitamin B_s	9	64B + 200 μg. B_s per 100 gm. diet	1	8		
		64B + 200 μg. B_s per 100 gm. diet	2	6		
		64B from 2 days before parturition	3	12		
		64B + 200 μg. B_s per 100 gm. diet	4	10		
	10	64B + 200 μg. B_s per 100 gm. diet	1	12		
		64B from 2 weeks before mating	2	10		
		64B + 200 μg. B_s per 100 gm. diet	3	11		
Pregnant stock ♀	11	64B from 4 days before parturition	1	8	0.1 cc. corn oil 15th, 17th and 22nd day	1
		64B + B_s from 2 weeks before mating	2	5		
Pregnant stock ♀	12	65 from 2 days before parturition	1	8		
Pregnant stock ♀	13	69 + 40 μg. B_s daily from 6 days before parturition	1	15		
		69 from 7 days before parturition	2	10		

[1] Number of young studied is less than number born in many litters because of usual de occurring soon after birth or reduction of size of large litters depending on condition of mot

[2] S

TABLE 3

Records of typical animals.

PORTION OF THE LITTER RECEIVING SUPPLEMENT		PORTION OF LITTER RECEIVING NO SUPPLEMENT		STOCK YOUNG SUCKLED BY FEMALE ON EXPERIMENTAL DIET	
Number of young	Results	Number of young	Results	Number of young	Results
		8	Died 11th–13th day		
2	Normal	2	Convulsions, died 17th–18th day	2	Convulsions — survived
		8	Normal		
		4	Normal		
2	Died 14th and 15th day	8	Died 12th–13th day		
		5	Died 15th–17th day		
4	Normal	2	Convulsions, 1 died 15th day	4	Convulsions — survived
1	Survived				
1	Survived				
		10	Normal		
		4	Died 13th day		
3	Normal			5	Convulsions — died
1	Survived — normal				
2	Died				
4	Died 11th–13th day	4	Died 11th–13th day		
4	Died 17th day	4	Died 13th–17th day		
4	Died 15th–17th day	2	Died 13th day		
8	Normal				
		5	Died 12th–19th day		
2	Survived — normal	8	6 died 17th–24th day		
		8	Normal		
		6	Normal		
		9	Died 19th–25th day		
		10	Normal		
		8	Normal		
		10	Died 13th–17th day		
		9	Normal		
1	Died 36th day	7	6 died 15th–21st day		
			7th died 41st day		
		5	Normal		
		8	6 died 15th–22nd day		
		10	Normal		
		10	Died 17th–47th day		

[1] Second generation on diet 22E.

to the young. The diet of the mother supplemented with pyridoxine was also found to be effective in preventing the onset of the symptoms. Such administration of pyridoxine to either mother or young allowed normal development of the young.

Pregnant females placed on diet 64B a short time before parturition were found to produce normal litters. All of the young, however, developed pyridoxine deficiency characterized by the convulsive symptoms. None of these animals survived the deficiency state unless the diet was supplemented with pyridoxine. Hemoglobin determinations on the blood of young animals in the acute stage of the deficiency indicated a normal hemoglobin level. By alternately feeding diet 64B and 64B supplemented with 40 μg. of pyridoxine daily in succeeding pregnancies to the same female it was possible to produce at will the characteristic deficiency in the young in one litter followed by a normal litter in the next reproductive cycle. The same results were obtained by alternating in the same manner the stock colony diet with diet 22E. It was possible as well to produce these convulsive symptoms in the young borne by females transferred during late pregnancy from the stock diet to diets 65 and 69. These were diets in which the liver extract had been omitted.

It was found that the occurrence of the deficiency symptoms was in some cases somewhat delayed in the young from stock females placed on the experimental diets after pregnancy was well advanced. It was also noted that a few of the young from such stock females changed to diet 22E, after exhibiting characteristic deficiency symptoms, survived on the unsupplemented diet. This would serve to indicate that diet 22E contains a borderline level of pyridoxine. Those animals which survived and which were maintained to maturity on diet 22E were found capable of normal reproduction but their young failed during the lactation period. The litters were not as large and the care of the young not as good as in the parent group and the young of five such animals all exhibited the characteristic convulsions and all failed to survive.

To determine whether these symptoms were a specific result of pyridoxine deficiency in the young, pregnant females were placed shortly before parturition on diet 65 modified to contain pyridoxine but deficient in riboflavin or pantothenic acid. Pantothenic acid deficiency, which occurred in the young between the tenth and twentieth day, was characterized by appearance of watery eyes and by sudden respiratory failure with collapse and death. The riboflavin-deficient young, on the other hand, became denuded and, although very much stunted in growth, were able to survive 5 to 6 weeks before extreme emaciation and death occurred. The symptoms exhibited by the young on either of these diets were in no way similar to those resulting from pyridoxine deficiency.

DISCUSSION

The authors are unaware of any previous report of characteristic symptoms of pyridoxine deficiency involving a paralytic or convulsive type of response in young rats of suckling age. This condition is readily reproducible and it has been demonstrated that pyridoxine is specific in its cure and prevention. Chick, Macrae, Martin and Martin ('38) observed epileptic fits in young pigs reared on synthetic diets and found that the condition could be cured with addition of the eluate fraction of yeast known to contain vitamin B_6. In 1940 a report from the same laboratory (Chick, El-Sadr and Worden) described occurrences of epileptiform fits in mature rats maintained for long periods on a diet deprived of vitamin B_6. Jukes ('39) reported the occurrence of spasmodic convulsions in chicks receiving vitamin B_6-deficient diets. Convulsions have been observed in dogs and have been attributed to a lack of vitamin B_6 (Fouts and coworkers, '38). It was interesting, in view of the occurrence of a microcytic hypochromic anemia in dogs on vitamin B_6-deficient rations (Fouts and coworkers, '38, '39), that our preliminary examination indicated no reduction in hemoglobin level in young rats during the acute stage of the deficiency. Jukes ('39) reported normal differential blood-cell counts for vitamin B_6-deficient

chicks. Woolley ('40) described a convulsive type of syndrome in mice fed highly purified diets which was alleviated by administration of pantothenic acid. The studies reported here, demonstrating a lack of relationship of pantothenic acid to the convulsive syndrome in suckling rats, illustrate further metabolic differences in this regard.

A neuromuscular syndrome in young rats, caused by a lack of alpha-tocopherol, has been reported by a number of investigators since the first description by Evans and Burr ('28). Our use of alpha-tocopherol in attempts to find a curative agent for the convulsive syndrome gave negative results and led us to conclude that we were not dealing with a vitamin E deficiency although the possibility was suggested that the alpha-tocopherol administered was not assimilated. To avoid a possible destructive action of rancidity, as shown by Commings and Mattill ('31) and discussed by Mattill ('38), the autoclaved peanuts were omitted from diet 22E without effect. In view of a recent report of MacKenzie et al. ('41), our levels of alpha-tocopherol were sufficient to counteract any dystrophic effect (Davis, Maynard and McCay, '38) of cod liver oil.

A relationship between fat metabolism and pyridoxine requirement has been suggested by a number of investigators. Using small amounts of corn oil, Quackenbush et al. ('39) obtained cure of a rat acrodynia similar to that described by György ('35). We were unable to demonstrate any curative effect of corn oil fed to young in the acute stage of the convulsive syndrome, although the survival period was slightly increased in some animals. These results furnish little evidence of a fat-sparing action on pyridoxine.

There was no observable injury to females maintained on diets devoid of pyridoxine during the period of pregnancy and lactation. After the loss of two or more litters from pyridoxine deficiency it was possible for such mothers to rear normal litters on these diets supplemented with pyridoxine. This dietary factor was readily transmitted to the young during lactation since inclusion of this factor in the

diet of the mother proved to be as effective as direct administration to the suckling young.

The young animals receiving pyridoxine directly developed and grew normally during the suckling stage, demonstrating that the quality, rather than quantity, of milk secreted was affected. This would indicate that pyridoxine can in no sense be designated a lactation factor. That small reserves of this factor are available to the animal on an unrestricted diet is brought out by the difficulty of the lactating female in secreting milk of a normal pyridoxine content after a relatively short period on a diet subminimal in pyridoxine, as well as by the rapidity with which the deficiency syndrome appeared in stock young transferred to mothers on a pyridoxine-deficient diet. The data presented are insufficient to indicate the minimum requirement of pyridoxine for suckling young or for secretion of milk of normal composition.

SUMMARY

A description is given of a convulsive syndrome which occurred in young rats during the suckling stage when being nursed by mothers on a diet considered to be satisfactory from the standpoint of growth and reproduction. This syndrome was cured or prevented by administration of crystalline pyridoxine and, by the use of suitable diets, was demonstrated to be a specific result of pyridoxine deficiency.

Rapid depletion of pyridoxine reserves was obtained in suckling young given to deficient mothers and lactating females transferred from a stock diet to one deficient in pyridoxine.

No injury was observed in females maintained on pyridoxine-restricted diets through the period of pregnancy and lactation. Under such conditions lactation was not impaired although the pyridoxine content of the milk was reduced to such extent that the convulsive syndrome developed in the young.

The authors wish to acknowledge the valuable technical assistance of Leo Friedman in this work.

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ANTIRACHITIC PROPERTIES OF A.T. 10 FOR THE RAT AND CHICKEN

JOHN T. CORRELL AND E. C. WISE

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

(Received for publication, August 27, 1941)

The common assumption is that dihydrotachysterol is the active component of A.T. 10 (Antitetanisches Präparat Nr. 10). However, since there is no certainty as to the exact composition of this product we shall follow the example of Shohl and Farber ('41) and refer to the material under discussion simply as A.T. 10.

Until recently, most of the experimental work on A.T. 10 was carried out in Germany. Early literature pertaining to this subject has been adequately reviewed by Albright, Bloomberg, Drake and Sulkowitch ('38). The first investigations with A.T. 10 revealed its power to increase serum calcium and relieve tetany after removal of the parathyroid glands. Most of the original investigators did not find A.T. 10 to be antirachitic. Harnapp ('35) was one experimenter who claimed that A.T. 10 had an antirachitic effect.

Albright and collaborators ('38, '39) published a series of clinical investigations comparing the effect of vitamin D_2, parathyroid extract, and A.T. 10 on calcium and phosphorus metabolism in idiopathic hypoparathyroidism, and in rickets. They concluded that A.T. 10 resembled vitamin D in its action by increasing calcium absorption from the gut and phosphorus excretion in the urine. But since it markedly raised serum calcium in the hypoparathyroid patients, and increased urinary phosphates more than did vitamin D, the authors decided that its action more nearly resembled that

of the parathyroid hormone. They suggested that possibly the reason A.T. 10 had not been found to be antirachitic was because of the increase in phosphorus excretion in the urine which it caused.

Recently, Shohl and Farber ('41) reported that they had confirmed the results of Harnapp and found A.T. 10 to be antirachitic for rats. In their studies the prophylactic type of feeding tests was employed and a high calcium, low phosphorus diet was used. They compared the action of vitamin D_2 and A.T. 10 and concluded that, under the conditions of their experiments, A.T. 10 was one four-hundredth as effective as vitamin D_2 in the curing of rickets and was five times as toxic.

With the appearance of the studies by Albright et al. the question arose in our laboratory as to what effect A.T. 10 would have on the serum phosphatase when administered to chickens. Correll and Wise ('38) have demonstrated that certain antirachitics are more efficient than others in maintaining a low serum phosphatase level in chicks. A substance with little or no vitamin D activity would not keep the serum phosphatase content normal but would permit the values to rise. In regard to the relation of parathyroid hormone to serum phosphatase, it has been reported that in the dog (Cantarow, Brundage and Housel, '37) and in man (Gutman, Tyson and Gutman, '36) the concentration of this enzyme is augmented by administration of parathyroid extract or by clinical hyperparathyroidism. Thus, if there was found to be an elevated serum phosphatase after administration of A.T. 10 to chicks it would be a further indication that A.T. 10 more nearly duplicated the action of parathyroid extract than that of an antirachitic agent.

The dual assay for vitamin D, with both rats and chickens, has become a recognized physiologic technique for differentiating between antirachitic substances. As a result of the paper by Shohl and Farber ('41), it was decided to determine what vitamin D potency A.T. 10 might indicate when assayed with rats by the standardized U.S.P. XI ('39) procedure. Using

the rat assay as a measure of antirachitic strength, we then decided to compare the response of the chick to various levels of vitamin D from cod liver oil, vitamin D_2, and A.T. 10 as manifested by body weight, bone ash, serum calcium, phosphorus and phosphatase.

The results obtained from these rat and chick investigations are submitted in this paper.

RAT EXPERIMENTS AND RESULTS

The rat assays to determine the antirachitic activity of A.T. 10 were carried out in a routine procedure conforming exactly to the U.S.P. XI ('39) technique for the assay of vitamin D. Final interpretations were made on the basis of the line test as described by Bills, Honeywell, Wirick and Nussmeier ('31). The antirachitic potency reported for A.T. 10 in this paper was based on these histologic readings, but in addition, in all but the first experiment, ash determinations for each group were made on pooled left tibiae, alcohol- and ether-extracted and moisture free. Also a pooled sample of blood was collected from each group and the serum calcium values obtained using the procedure of Clark and Collip ('25).

Materials investigated were diluted with olive oil so that a definite number of drops supplied the desired daily supplement of active ingredient.

We used commercial preparations as sources of vitamin D_2 [1] and the A.T. 10.[2] The daily doses indicated in the tables represent the weight of active principle fed, calculated from the label statement for each product.

In the first experiment, table 1, we found vitamin D_2 to assay 40,000,000 I.U. per gram (group 89) which is the accepted potency of this material. When A.T. 10 was fed on a basis of one four-hundredth the potency of vitamin D_2 (group 90), as suggested by the work of Shohl et al., little curative effect was found as measured by the line test.

[1] Drisdol. Winthrop Chemical Company, New York.

[2] Hytakerol. Winthrop Chemical Company, New York.

TABLE 1

Antirachitic potency of A.T. 10 and vitamin D_2 as measured by the U.S.P. XI rat assay.[1]

ASSAY NO.	MATERIAL ASSAYED	DAILY DOSE FOR 6 DAYS	DEGREE OF HEALING	POTENCY FOUND PER GRAM	FINAL WEIGHT	INORG. Ca/100 cc. OF SERUM	BONE ASH
			line test	*I.U.*	*gm.*	*mg.*	*%*
Experiment I							
92	U.S.P.[2] reference oil no. 2	6.96 mg.	1.9+	115	69.0		
89	Vitamin D_2[3]	0.02 μg.	2+	40,000,000	75.0		
90	A.T.10	8.0 μg.	0.3+	Dose too small	68.0		
Experiment II							
127	Negative controls	0	0.2+	0	79.0	10.1	34.3
126	U.S.P.[2] reference oil no. 2	6.96 mg.	2.0+	115	75.0	11.8	34.0
121	A.T. 10	13.3 μg.	0.6+	Dose too small	73.0	11.5	37.2
122	A.T. 10	26.6 μg.	2.0+	30,000	76.0	11.6	37.0
Experiment III							
229	Negative controls	0	0	0	71.5	8.2	30.3
147	Positive controls[4]		Complete calcification		146.4	9.0	58.1
228	U.S.P.[2] reference oil no. 2	6.96 mg.	2.0+	115	74.4	10.0	33.7
227	A.T. 10	26.6 μg.	1.9+	30,000	78.8	10.1	34.1
226	A.T. 10	78.0 μg.	3.1+	No toxicity	76.9	10.2	37.3

[1] Values represent pooled samples from eight to ten animals per group.
[2] Standardized, labeled, potency of 115 I.U. per gram.
[3] Expected potency of 40,000,000 I.U. per gram.
[4] Raised on an adequate diet.

Higher levels of A.T. 10 were administered in the second and third experiments. At 13.3 μg. daily (group 121), the compound again failed to show adequate calcification in the epiphysis of the bones. However, when given at a level of 26.6 μg. (groups 122 and 227), a good 2 plus healing was observed, comparable to that obtained from the reference oil (groups 126 and 228). This indicated a vitamin D activity for A.T. 10 of 30,000 I.U. per gram or approximately 1/1300 that of vitamin D_2 as assayed by the U.S.P. XI curative technique.

No toxicity was noted at a higher level fed, 78.0 μg. (group 226); these animals simply demonstrated a greater degree of calcification. The ash content of the bones in all the groups was low when compared with normal rats of that age (group 147), indicating that in this type of assay recalcification of the rachitic bones had not gone very far during the short test period. The amounts of A.T. 10 administered to these rats had no significant effect on raising the serum calcium values above normal.

CHICK EXPERIMENTS AND RESULTS

Single comb White Leghorn chicks were started on assay when 1 day old (weight 30 to 35 gm.) and the experiments terminated on the twenty-first day. Care of the animals, basal diet employed, and details of experimental technique were essentially as described in earlier papers (Correll and Wise, '38; Correll, '41). All values in the tables represent pooled samples from at least fifteen birds for each group.

The oils that were tested were diluted with sesame oil and adjusted to bring the total oil supplement of the feed to 1%. Vitamin D_2 dilutions were made with propylene glycol, the vehicle employed in the commercial preparation used. Again the solutions were calculated to be of such potency that the final test ration would be 1% propylene glycol. In the experiments comparing vitamin D_2 and A.T. 10, all diets including the controls were made identical by the addition of both 1% sesame oil and 1% propylene glycol, with or without the

active principles as the study required. The cod liver oil fed had a potency of 150 I.U. per gram.

The first experiment was planned to compare the response of the chick to A.T. 10 and vitamin D_2 on a weight and unit basis, and to determine the physiologic activity of several levels of A.T. 10 in the birds. In our hands ('38), chicks on this type of experiment require around 25 I.U. of vitamin D from cod liver oil per 100 gm. of diet for minimum protection. Hence, one such group was included to serve as positive controls.

Table 2, experiment I, presents the results of this research. Vitamin D_2, when fed at a level of 2.5 μg., or 100 I.U. per 100 gm. of diet (group 437), gave no protection to the birds, but at a level of 25 μg., 1000 I.U. (group 438), the bone ash values obtained were as high as those of the reference group 432 and the phosphatase values nearly as low. Such a dose of vitamin D_2 approaches closely the minimum requirement. In contrast to this, A.T. 10 fed at 20 μg., 0.6 I.U. per 100 gm. of diet (group 433), or at nearly equal the weight of vitamin D_2 found effective, failed to elicit any antirachitic response. A.T. 10 at 50 μg., 1.5 I.U. (group 440), demonstrated some protection as seen by the bone ash, serum calcium and phosphatase values obtained. When supplemented into 100 gm. of ration in the amount of 200 μg., 6 I.U. (group 434), A.T. 10 afforded complete protection against rickets for the birds. In consideration of the responses obtained from some preliminary experiments and group 440, table 2, this dose of 200 μg. or 6 I.U. appears to be very close to the minimum protective dose for A.T. 10. Thus, on a weight basis, A.T. 10 is about one-eighth as effective for the chick as vitamin D_2. When compared, however, on a rat unit basis, A.T. 10 is nearly 200 times more beneficial than vitamin D_2.

Some indication of toxicity for the chick from A.T. 10 was noted in group 435, both from the appearance of the birds and the lowered final weight. The larger dose fed to group 436 was definitely deleterious. For chickens the toxic dose

TABLE 2

Comparison of the antirachitic effect of A.T. 10, vitamin D_2, and vitamin D from cod liver oil in chicks.

ASSAY NO.	MATERIAL ASSAYED	DOSE PER 100 GM. OF DIET	FINAL AVERAGE WEIGHT	INORG. Ca/100 CC. OF SERUM	INORG. P/100 CC. OF SERUM	PHOSPHATASE PER 100 CC. OF SERUM	BONE ASH	INTERPRETATION
			gm.	*mg.*	*mg.*	*units*	%	
Experiment I								
431	Negative controls	0	123	7.0	6.6	120	38.0	Rachitic
432	Cod liver oil	166 mg. 25 I.U.	142	9.7	7.5	50	46.6	Normal
437	Vitamin D_2	2.5 μg. 100 I.U.	115	7.3	6.7	114	38.3	Rachitic
438	Vitamin D_2	25.0 μg. 1000 I.U.	142	9.5	7.9	62	46.3	Normal
433	A.T. 10	20 μg. 0.6 I.U.	143	7.0	6.6	117	37.7	Rachitic
440	A.T. 10	50 μg. 1.5 I.U.	132	9.1	6.5	75	44.5	Substantial protection
434	A.T. 10	200 μg. 6 I.U.	151	9.8	7.1	49	48.5	Normal
435	A.T. 10	1.0 mg. 30 I.U.	112	11.0	6.7	37	47.1	Slight indication of toxicity
436	A.T. 10	3.0 mg. 90 I.U.	89	11.2	5.9	30	44.9	Toxicity indicated
Experiment II								
441	Negative controls	0	136	4.3	5.5	169	33.6	Rachitic
444	Cod liver oil	20 mg. 3 I.U.	138	5.7	6.1	122	35.5	Slight protection
443	Cod liver oil	40 mg. 6 I.U.	141	5.1	6.2	135	35.2	Slight protection
442	Cod liver oil	166 mg. 25 I.U.	147	8.0	6.9	66	45.4	Normal
447	A.T. 10	50 μg. 1.5 I.U.	140	6.3	6.1	99	38.5	Definite protection
446	A.T. 10	100 μg. 3 I.U.	152	6.7	6.6	103	42.0	Substantial protection
445	A.T. 10	200 μg. 6 I.U.	150	9.1	7.3	47	47.5	Normal

of A.T. 10 is apparently about five times the minimum protective level.

Serum calcium concentration of these chicks with normal parathyroid glands was not significantly altered except where the levels of administered A.T. 10 tended to be morbific. In such groups there was a rise in the calcium values.

As a result of this chick experiment the data revealed an unexpected development. On a rat unit basis, apparently A.T. 10 was even more effective in maintaining high bone ash, low serum phosphatase, and normal serum phosphorus and calcium values, than was the vitamin D from cod liver oil itself. A final investigation was organized to compare more exactly the relative efficacy of these two antirachitics for the chick. In table 2, experiment II, the results of this study are recorded.

In agreement with previous results, vitamin D from cod liver oil at a level of 25 I.U. per 100 gm. of ration (group 442) proved adequate to protect the chicks from rickets. In this particular assay the dose appears to be border line as the serum calcium and bone ash values are a trifle lower than usual and the phosphatase slightly elevated. This only strengthens the comparison and categorically demonstrates that 6 I.U. from A.T. 10 (group 445) is as effective as 25 I.U. from cod liver oil. Further support is lent to this conclusion by comparing the results obtained from 6 I.U. of vitamin D from cod liver oil (group 443), which evidenced but slight protection, with the normal values found when 6 I.U. were given from A.T. 10 (group 445). A.T. 10, at a level of 3 I.U. per 100 gm. of diet (group 446), demonstrated substantial protection while the same dose from cod liver oil (group 444) was only slightly better than the negative controls (group 441). In fact, 1.5 I.U. from A.T. 10 (group 447) was more effective than 6 I.U. from cod liver oil (group 443). Under the conditions of these experiments, rat unit for rat unit, A.T. 10 was four to five times more efficacious as an antirachitic for the chick than was the vitamin D from cod liver oil.

DISCUSSION

There is little published data concerning the antirachitic activity of A.T. 10. The material has been standardized on the basis of its toxicity for mice. Using an entirely different technique than the one employed in this work, Shohl and Farber ('41) concluded that in their experiments A.T. 10 was one four-hundredth as potent as vitamin D_2. No attempt was made to state its antirachitic capacity in units. However, vitamin D_2 has an established strength of 40,000,000 I.U. per gram so those studies which found A.T. 10 to be one four-hundredth as active, circumstantially indicate A.T. 10 to have a potency of 100,000 I.U. per gram on the basis of the protective technique.

No difficulty was experienced in this laboratory in finding a level of A.T. 10 which, when assayed on rats in exact accordance with the U.S.P. procedure for measuring vitamin D activity, would repeatedly yield results comparable to those obtained from the reference oil. At this level A.T. 10 indicated an antirachitic strength of 30,000 I.U. per gram. That is, by the curative method a rat required 26.6 μg. daily of A.T. 10 to show a 2+ healing; 0.02 μg. of vitamin D_2 gave the same response. In the protective method of Shohl and Farber 25 μg. of A.T. 10 prevented rickets and 0.0625 μg. of vitamin D_2 yielded comparable results. Thus with the curative procedure A.T. 10 has one thirteen-hundredths the antirachitic effect of vitamin D_2 as contrasted to the one four-hundredths indicated by preventive feeding. Since the primary purpose of our study was to investigate the possible antirachitic activity of A.T. 10 for the chick, the rat studies were limited to establishing its U.S.P. potency without reference to toxicity.

In a prophylactic type of experiment with chickens, A.T. 10 was found to afford a minimum protective dose when mixed into the ration at a level of approximately 200 μg. or 6 I.U. per 100 gm. of diet. Again detailed toxicity studies were reserved for future investigation and are incidental to this report. However, in feeding the higher amounts of A.T. 10 to the chicks, it was observed that such groups demonstrated lowered

final weights and ragged gross appearance. Using these criteria, a toxic dose was noted at a level of about 1 mg. or 30 I.U. per 100 gm. of diet. Thus the protective dose was one-fifth the toxic dose for chicks and agrees with the findings of Shohl and Farber ('41) who used rats. Vitamin D_2 was not administered at levels high enough to manifest any toxicity; if the response of the chick were again similar to that reported for the rat it should require around 10,000,000 I.U. of vitamin D_2 per 100 gm. of ration to be deleterious.

About 1000 I.U. of vitamin D_2 per 100 gm. of ration served to protect the birds from rickets. It was, therefore, only one one hundred-sixtieth as effective an antirachitic for chicks as A.T. 10. This observation, that vitamin D_2 was so much less efficient than A.T. 10 in preventing rickets in chicks, is strong evidence that the action of A.T. 10 is not due to contamination with vitamin D_2.

A remarkable fact that shows up in these data is that 6 I.U. of A.T. 10 were as beneficial for the chicks as 25 I.U. from cod liver oil. As far as we are aware this is the first report of an antirachitic material of plant origin demonstrating in chickens anywhere near the efficiency of vitamin D from cod liver oil when compared on a rat unit basis. Many substances have been subjected to the dual rat-chick analysis. Some of these from animal origin have proved equally efficient, and a few more efficient for the chick than vitamin D from cod liver oil (Bills, Massengale, Imboden and Hall, '37). In contrast to this, Grab ('36), among others, reported studies on a whole series of plant sterols, not one of which even approached antirachitic substances of animal origin in relative efficacy for the birds.

The picture is further complicated by the realization that the plant sterol A.T. 10, supposedly dihydrotachysterol, is a reduction product of irradiated ergosterol and hence chemically very closely related to vitamin D_2. Yet vitamin D_2 is only about one-fourtieth as effective, rat unit for rat unit, as vitamin D from cod liver oil on the chick, while a rat unit

of A.T. 10 is four or five times more efficacious than one from cod liver oil.

The doses of A.T. 10 fed did not influence serum calcium values so that they differed from those obtained with the reference oils, except in the two groups of chicks which were offered the largest quantities. This is additional evidence that A.T. 10 will only demonstrate an elevation in serum calcium when administered to parathyroidectomized animals; or to normal animals with functioning parathyroids in amounts massive enough to be harmful. It will also be noted that, similar to the action of an antirachitic, increasing quantities of A.T. 10 resulted in decreasing phosphatase values, instead of causing a rise as has been reported for parathyroid hormone. Thus, within the limitations of these experiments the biologic action of A.T. 10 resembled that of vitamin D rather than that of the parathyroid hormone.

SUMMARY

1. A.T. 10 has an antirachitic potency of 30,000 I.U. per gram as assayed by the U.S.P. XI rat technique.

2. A minimum protective level of A.T. 10 against rickets for chicks is around 6 I.U. of antirachitic activity per 100 gm. of ration. A toxic level is apparently about five times this dose.

3. By weight comparison A.T. 10 is approximately one-eighth as potent an antirachitic for chicks as vitamin D_2. However, on a rat unit basis, A.T. 10 is nearly 200 times more effective than vitamin D_2.

4. Rat unit for rat unit, A.T. 10 is about four times more powerful an antirachitic on the chick than vitamin D from cod liver oil. This is the first time an antirachitic substance of supposedly vegetable origin has demonstrated in chickens anywhere near the rickets preventive ability of vitamin D from cod liver oil when compared on a rat unit basis.

5. Under the conditions of these experiments the action of A.T. 10 resembles that of vitamin D rather than parathyroid hormone.

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THE IRON METABOLISM AND REQUIREMENT OF YOUNG WOMEN [1]

RUTH M. LEVERTON AND ALICE G. MARSH
Human Nutrition Laboratory, Department of Home Economics, University of Nebraska, Lincoln

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Recent developments in the field of iron research have challenged the long accepted "balance" study as an adequate method for investigating the iron metabolism and requirement of humans. When iron requirements were formulated from such studies it was assumed that the excretion reflects the body's need or daily use of iron and that this need is measured by the difference between the intake and outgo. When interpreted in this way an excretion which equalled the intake indicated that the body's need was just being met, if the excretion exceeded the intake the need was not being met, and if the excretion was exceeded by the intake iron was being stored. It now appears, however, that iron excretion is not a criterion of the body's need or utilization of iron but rather a reflection of the rate or completeness of absorption and that many factors other than need may influence absorption.

McCance and Widdowson ('38) have reported that when 7 mg. of iron daily were given intravenously for 2 weeks to six adults already in iron equilibrium their level of excretion did not rise. They present this observation as the culminating evidence for the new theory that the intestine has no ability to regulate by excretion the amount of iron in the body and that the iron found in the feces is only that which has escaped

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absorption rather than iron which has been absorbed, utilized, and re-excreted.

Hahn et al. ('39) injected five dogs with radioactive iron and from the metabolism results concluded that the iron stores of the body are controlled by absorption or lack of absorption from the intestine rather than by excretion into the intestine.

Further evidence in support of this hypothesis was presented by Leverton ('41) in a study planned to determine whether the fecal excretion of iron would ever significantly exceed the intake, regardless of how low the intake became. Four healthy college girls were kept for several months on a diet that contained only 3.5 mg. of iron daily, but was adequate in other respects. Their average fecal excretion of iron did not exceed the daily intake, thus indicating no elimination of iron through the walls of the intestine.

With iron, unlike other minerals, more than a state of equilibrium is to be desired in young women; there must be storage to replace the iron lost in the menses. This means that regardless of the theories of excretion metabolism results must show positive balances if optimum nutrition in respect to iron is to be maintained.

Barer and Fowler ('36) have found the iron loss during normal menstruation to approximate 20 mg. This would require an average daily storage of 0.7 mg. during a 28-day menstrual cycle to prevent a gradual depletion of body iron. As a recommendation, however, a figure somewhat above the average, at least 1 mg. a day, would be advisable to insure some margin to cover the individual variations in losses greater than 20 mg.

The iron work to be reported here was part of a study of the mineral and nitrogen metabolism of young women planned to determine their self-chosen intakes and subsequent balances of calcium, phosphorus, iron, copper and nitrogen. It was particularly hoped that information would be secured which would clarify the interrelationship of the needs for different nutrients as well as furnish additional bases for dietary recommendations for this age group.

PROCEDURE

Between the fall of 1937 and the spring of 1940 ninety-nine 1-week metabolism studies were made of sixty-nine women students who were between the ages of 16 and 27 years and were living on self-chosen diets. Fifty-two subjects were studied once; seven, twice; seven, three times; and three, four times. The repeat studies were made 6 months to a year apart so that a girl was in a different age group each time she was studied. All of the girls were in normal health as judged by a general medical examination and critical inspection. Studies were made during the inter-menstrual periods. Most of the girls were doing light housekeeping, some were living at home, and a very few ate at the college cafeteria.

A real effort was made to enable the subjects to continue their usual mode of living and dietary habits during the week of study. Each girl was told that the study to be made on her was not one of correct or incorrect dietary practices, but rather of how much of the food she ordinarily ate was stored in her body, and thus she realized that the success of the study depended primarily upon her continuing her usual dietary and living habits. Her accuracy, dependability, and careful cooperation were solicited but she was also encouraged to live in her usual manner as casually as possible.

All of the food eaten was weighed and sampled for analysis and the feces and urine were collected for the period of study. Carmine was given to mark the stools at the beginning and end of the week. The aliquot food samples were combined into a 1-week composite as were the stools and also the daily urine collections. As previously described (Leverton, '41), the separate composites of foods and feces were made into brown digests with 20% hydrochloric acid. Aliquots of the brown digests were oxidized and iron was determined as the thiocyanate. Every care was taken to prevent contamination of the materials during or following collection, and the analytical work was done in a laboratory especially equipped to preclude iron contamination. Calcium, phosphorus, nitrogen, and copper determinations were also made.

RESULTS

Because the greatest value of these results lies in the number of studies and, therefore, the cross-section of metabolic activity that they represent for young women of this age, average figures will be given greater emphasis than individual ones.

The average intake, excretion, and storage of iron for the whole group are given in table 1.

TABLE 1

Intake, excretion, and storage of iron

SOURCE OF DATA	IRON DAILY		
	Intake	Excretion	Storage
	mg.	*mg.*	*mg.*
69 studies of 69 individuals	10.71	9.38	1.33
99 studies of 69 individuals	10.44	9.07	1.37

These average daily intakes of iron permitted a liberal storage for replacement of menstrual loss. The slight drop in intake and excretion values when the data from the repeated studies are included in the averages is probably not significant, especially since the amount of iron that was stored was practically the same in both cases.

In order to analyze differences and still deal with a group of subjects rather than individual cases the studies have been sorted into five groups according to the level of iron intake of the individual cases.

Table 2 contains the average intake and excretion of iron of the subjects in each group accompanied by the average of the heights, weights, and ages of each group. While individual daily intakes of iron range from 5.94 to 16.71 mg., sixty-two of the ninety-nine values fall between 8.00 and 11.99 mg.

In no group did the average of the daily excretions exceed the average of the daily intakes of iron to result in a so-called negative balance. More important than this, however, is the decrease in the percentage of individual cases of negative balances and the steady increase in the amount of iron stored

as the level of intake rose. These two facts indicate that for average individuals in general the long-accepted relation between iron intake and iron absorption still holds. The average storage of the group with the lowest daily intake, 5.94 to 7.99 mg., was not great enough to cover normal menstrual losses, as was the case for the other groups.

TABLE 2

Average of iron metabolism data for ninety-nine studies according to level of iron intake

RANGE OF DAILY INTAKE OF IRON	NO. STUDIES	IRON DAILY			IN NEGATIVE BALANCE	AVERAGE			
		Intake	Excretion	Storage		Ht.	Wt.	Age	
mg.		*mg.*	*mg.*	*mg.*	%	*cm.*	*kg.*	*yr.*	*mo.*
5.94– 7.99	15	7.16	6.75	0.41	40	160.8	53.7	20	10
8.00– 9.99	31	9.18	8.21	0.96	23	162.1	56.0	20	7
10.00–11.99	31	10.90	9.53	1.34	19	164.3	56.8	20	7
12.00–13.99	15	12.91	11.15	1.77	13	164.8	58.8	21	11
14.00–16.71	7	15.85	11.30	4.55	0	163.9	58.2	20	7

Previous publications from this laboratory present evidence that dietary essentials other than iron are important factors in preventing or correcting simple anemia in young women. For this reason in table 3 the diets of all subjects who were storing iron and the diets of all the subjects in negative balance are compared with respect to iron, calcium, phosphorus, and nitrogen. While these nutrients are not the only ones by which to judge the adequacy of the diets they are recognized as important objective criteria.

The consistency with which the differences are always in favor of the group that is storing iron is probably of greater significance than are the actual numerical differences between content of the diets of the two groups.

These numerical differences are greatly accentuated, however, at levels of iron intake below 8 mg. This is also shown in table 3 when the fifteen individual studies in which the daily iron intakes ranged from 5.94 to 7.99 mg. are similarly divided into those storing iron and those not storing it.

These figures of iron intake and balance show the limitations to the value of average figures. In table 2 the group with this low intake, 5.94 to 7.99 mg., did not have a storage sufficient to cover menstrual losses but here the figures show that those who were storing iron did so to a greater extent than 1.37 mg. which was the average for all ninety-nine studies.

TABLE 3

Comparison of intakes of iron, calcium, phosphorus, and nitrogen

SOURCE OF DATA	AVERAGE DAILY					HEMOGLOBIN
	Iron		Ca	P	N	
	Intake	Balance	Intake			
	mg.	*mg.*	*gm.*	*gm.*	*gm.*	*gm./100 ml.*
For entire group:						
78 studies of 52 individuals storing iron	10.75	+2.11	0.88	1.36	9.70	13.3
21 studies of 17 individuals not storing iron	9.29	—0.82	0.77	1.13	8.64	12.8
For subjects with daily iron intakes below 8 mg.:						
9 studies of 7 individuals storing iron	7.21	+1.55	1.04	1.27	9.70	13.3
6 studies of 5 individuals not storing iron	7.09	—1.30	0.66	0.94	7.25	12.0

It would appear then from the differences in the calcium, phosphorus, and nitrogen content of the diets that when the daily iron intake of young women is below 8 mg. the adequacy of the diet in other nutrients, probably not just those discussed here, is the deciding factor in stimulating storage for replacement of that lost in menstruation and perhaps for other functions not so clearly understood or measurable. Several years of contact with the girls, and their families if the girls lived at home, leave no doubt in the minds of the authors as to the general superiority of the food habits and nutritional history of the seven girls who were storing iron at this low level of intake as compared with the five who were not.

This is specifically illustrated by the two cases shown in table 4 — subject 69 and subject 17 who were living at home and eating meals prepared by their mothers. Subject 69 had an unquestionably good dietary history and good food habits; although she cared little for meat she ate abundantly of milk and cheese, fruits and vegetables and used whole wheat bread and cereals. Subject 17 was not conscious of having poor food habits but had practically never drunk milk, did not care for vegetables and ate white bread entirely.

TABLE 4

Comparison of subjects 69 and 17

SUBJECT	AGE WHEN STUDIED		AVERAGE DAILY				
			Iron		Calcium intake	Nitrogen	
			Intake	Balance		Total	Per kilo
	yr.	*mo.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
No. 69 on the	17	1	7.47	+3.47	1.08	9.28	0.180
better diet	17	8	7.41	+1.75	1.17	11.28	0.221
	18	2	5.94	+0.77	1.08	9.32	0.182
No. 17 on the	19	7	7.89	—1.65	0.53	5.89	0.090
poorer diet	20	3	6.29	—1.02	0.44	6.95	0.116

DISCUSSION

If one accepts the premise that the intestinal tract does not re-excrete iron the significance of observed negative balances in iron metabolism studies is problematic. Their occurrence may be attributable to such factors as: (1) too short a period of study; (2) an atypical state of the subject; (3) difficulty in separating the carmine-marked and unmarked portions of the feces; and (4) an abnormal permeability of the individual's intestinal tract to the re-excretion of iron from the body. The same factors together with adsorption of iron by the intestinal mucosa could also operate to produce fictitious storages of iron. While these factors may apply to occasional individual cases it may be safely assumed that ninety-nine studies would give a typical cross-section of the iron metabolism of a group

of normal young women and show whether they were storing enough of this element to replace menstrual losses.

The practical significance of these results is the finding that the customary intake of iron of young women on self-chosen diets permits a storage of more than enough to replace the iron lost in the menses and that two-thirds of this amount would be sufficient if the diet were generous in other nutrients.

The figure of 10.44 mg., representing the average daily intake of the group and associated with a daily storage of 1.37 mg., is valuable in showing group performance but it overlooks the individual subjects who were in negative balance. In these cases of apparent negative balance, regardless of the level of intake, the correction would seem to be in the general improvement of the diet with larger quantities of protective foods rather than by an increased intake of iron alone.

This influence of the quality of the diet upon the absorption of iron, especially at low levels of intake, has been observed in other studies in this laboratory. When a group of college women with simple hypochromic anemia was given daily supplements of vitamin B complex or additional protein from dry milk, cheese, and peanuts the hemoglobin content of their blood increased even though no medicinal iron was given (Leverton and Marsh, '41). Also when four young women had a generous diet except that the daily iron intakes were between 3.5 and 4.5 mg. for several months they remained in excellent health and there was no great decrease in hemoglobin (Leverton, '41). When their iron intake was increased to 6.55 mg. their average daily storage was 2.44 mg.

The bearing of these findings upon the dietary recommendations for iron is less than might be expected. While the results show that the chances of storing iron are greater at higher levels of intake they also show that ample storage occurred at surprisingly low intakes when the diet was rich in other nutrients. A person's intake could be high in iron and poor in protective foods but if it were rich in protective foods it could hardly be deficient in iron — both because the protective

foods are good sources of iron and because the amount of iron required appears to be small if the supply of other nutrients is liberal. It follows then that a well-balanced optimum diet probably holds more promise than does iron medication for a permanent general improvement in the treatment of simple hypochromic anemia in young women.

SUMMARY AND CONCLUSIONS

Ninety-nine 1-week iron metabolism studies were made on sixty-nine young women between the ages of 16 and 27 years who were living on self-chosen diets.

The average daily intake of 10.44 mg. of iron provided an average daily storage of 1.37 mg. which is ample to replace normal menstrual losses. As the level of intake rose from below 8 mg. to 16 mg. the occurrence of negative balances decreased and the amount of iron that was stored increased.

Seven subjects whose diets were generous in other nutrients had an average daily iron intake of 7.21 mg. and were storing 1.55 mg. daily or more than enough iron to replace that lost in normal menstruation. Five other subjects with similar iron intakes but suboptimum diets in other respects had an average daily negative balance of 1.30 mg. of iron.

There is no occasion for suggesting more or less iron than is recommended in current dietary standards for young women. However, calculations to determine whether a diet contains an adequate amount of iron may eventually be discontinued since it appears that emphasis should rather be placed upon obtaining diets optimum in other essential nutrients which have been shown to function in efficient iron absorption and utilization.

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THE EFFECT OF ENZYMATIC DIGESTION ON THE PANTOTHENIC ACID CONTENT OF MEATS DETERMINED BY THE MICROBIOLOGICAL METHOD[1]

HARRY A. WAISMAN, LAVELL M. HENDERSON, J. M. McINTIRE AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

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In a previous publication (Waisman et al., '39), we reported the relative pantothenic acid potency of animal tissues as determined by bioassay with chicks maintained on a dermatitis producing ration. While these results furnished an approximation of the pantothenic acid in meat products the availability of a rapid microbiological method together with pure calcium pantothenate enabled us to determine the pantothenic acid content of many of the tissues previously assayed on a more quantitative basis. A large number of additional meat samples have also been assayed by this method.

Several microbiological methods have been proposed for the estimation of pantothenic acid. Williams and Saunders ('34) used the yeast growth method, while Pennington, Snell and Williams ('40) and Strong, Feeney and Earle ('41) used the organism Lactobacillus casei ε, and Pelczar and Porter ('41) used Morgan's bacillus.

METHOD

The organism and medium which were employed in this study were those described by Strong, Feeney and Earle ('41).

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Preliminary trials using their method of hot water extraction on animal tissues gave erratic results and it was found that homogenization of the sample in the Potter-Elvehjem ('36) homogenizer followed by the hot water extraction (autoclaving at the natural pH) gave more consistent results. However, the values obtained by this method were not entirely satisfactory. It appeared that an enzymatic digestion would make the pantothenic acid more readily available to the test organism. Although Pennington, Snell and Williams ('40) suggested the use of autolysis of the fresh tissue, we believed that a more thorough digest of the dried samples which we had at our disposal would be advisable.

Since the samples we used contained high amounts of protein, it seemed advisable to try pepsin for the digestion. The pepsin digestions were performed on both fresh and dried samples of the same tissue together with autolysis of fresh tissue at the natural pH. All samples were homogenized before being subjected to the particular extraction procedure. The pantothenic acid values obtained after these treatments are given in table 1. It will be seen that pepsin digestion of either the dried or fresh tissue gave higher results than the untreated fresh or dried tissue. The autolysis of the fresh tissues also produced an increased pantothenic acid value over that for the untreated fresh tissues in all the samples tested.

Further work on enzymatic digestion was carried out using clarase.[2] Several levels of clarase were used with the homogenized suspensions of the dried meat sample. Equal weights of clarase and meat were used as well as twice as much enzyme as meat, and in both cases the pantothenic acid titer was the same. However, the values obtained in all cases were higher than those after pepsin digestion.

The use of a pancreatic extract appeared to us to be a more desirable enzyme preparation to use because of its ability to give more complete liquefaction of the tissue. Several

[2] Obtained from Takamine Laboratories, 193 Arlington Avenue, Passaic, New Jersey.

trials with pancreatin,[3] as well as with clarase and pepsin, on pure calcium pantothenate satisfied us that the enzymes had no effect on the amide linkage of the vitamin and that the quantity of enzyme added for digestion furnished only in-

TABLE 1

Effect of enzymatic digestion and drying procedure on microbiologically determined pantothenic acid.

SAMPLE	MICROGRAMS PANTOTHENIC ACID PER GRAM						
	Fresh [1]				Dry [1]		
	Untreated	Autolyzed	Pepsin digested	Pancreatic digested	Untreated	Pepsin digested	Pancreatic digested
Veal heart 155	120	158	160		125	166	
Pork loin 156	25	30	33		27	35	
Beef round 157	13	16	19		13	21	
Veal liver 158	130	160	159		145	145	
Pork liver 159	159	193	177		145	194	
Pork loin 165				42			45
Beef heart 166				119			122
Beef kidney 167				242			223
Beef liver 168				290			283

[1] Expressed as micrograms calcium pantothenate calculated to the dry weight basis.

significant amounts of pantothenic acid. All the values were corrected by subtracting the amount of pantothenic acid in the enzyme preparation. The pancreatic digests were made in a buffered solution at pH 7.0 to 7.5 so that no pantothenic acid would be destroyed by either acid or alkali.

[3] Merck and Company, Rahway, New Jersey.

The marked increases in apparent pantothenic acid content after enzymatic digestion led to a consideration of the possibility that enzymatic treatment of the tissue liberated some bacterial growth-stimulating substance which accounted for the higher values obtained. After several tests involving numerous recovery experiments we were convinced that the higher results obtained after pancreatic digestion were actually due to increased liberation of pantothenic acid from its natural complexes.

Having assured ourselves that more consistent results could be obtained by enzymatic digestion, we determined whether the use of our dried tissues was satisfactory for the microbiological assays. Accordingly several samples of meat which were representative of both high and low pantothenic acid containing tissues were obtained from a local market. Part of each sample was assayed in both the fresh and dry state. After determining the moisture content of the fresh tissue it was possible to compare the amount of pantothenic acid in both fresh and dried portions on the same basis. As can be seen from the figures under "fresh" and "dry" in table 1, it was apparent that our drying procedure had no deleterious effect on the pantothenic acid content. The vitamin content of the samples tested in the dry state was equal to that of the fresh weight when the pantothenic acid content was expressed in micrograms per gram of the dried tissue.

The pepsin, clarase and pancreatic digestions were performed on the majority of our meat samples, but to conserve space only typical figures are given in table 2. It will be seen that greater amounts of pantothenic acid were obtained after clarase treatment than after pepsin digestion and a still further increase was obtained after pancreatic digestion. Although there were several samples which did not show this progressive increase after treatment with the respective enzymes, the majority of the muscular tissues did give the highest pantothenic acid values after pancreatic digestion. At present, we are satisfied that the most reliable pantothenic acid

figures for animal tissues can be obtained after pancreatic digestion of the homogenized tissue.

The exact procedure used in the microbiological assay of animal tissues using the pancreatic digestion is as follows: A half gram of the finely pulverized or homogenized sample is placed in a suitable brown bottle with a solution containing 50 mg. of pancreatin and 0.5 gm. of K_2HPO_4 and the volume

TABLE 2

Typical pantothenic acid values obtained after various enzymatic digestions on the dried meat.

SAMPLE	SAMPLE NO.	TREATMENT OF MEAT	DIGESTION WITH		
			Pepsin	Clarase	Pancreatin
			Micrograms per gram dried tissue [1]		
Beef liver	98	——	138	152	150
Beef liver	150	——	240	248	240
Veal liver	158	——	145	145	158
Pork liver	153	——	230	221	221
Beef kidney	81	——	158	178	173
Beef round	154	——	38	40	49
Pork ham	139	——	28	32	45
Pork ham	141	Fried	10	19	20
Pork ham	142	Roasted	7	14	27
Pork ham	117	Tenderized	16	32	40
Pork ham	102	Smoked	30	37	44
Pork loin	93	Fried	8	15	22
Beef tongue	82	——	34	—	74

[1] Expressed as calcium pantothenate.

adjusted to 100 ml. with distilled water. The mixture is incubated for 24–72 hours at 38° C. with 1 ml. of toluene added as a preservative. After digestion a suitable aliquot is removed and diluted so that the pantothenic acid content falls in the range required by the assay method. The medium, culture, inoculation, and titration procedures of the assay are essentially those reported by Strong, Feeney and Earle ('41). Table 3 includes the pantothenic acid values for more than eighty samples of animal tissue.

TABLE 3

Microbiologically determined pantothenic acid of pancreatic digested animal tissues.

TISSUE		MICROGRAMS PER GRAM [1]	
		Dry	Fresh
Beef liver	98	150	—
	131	254	74
	150	240	66
	163	208	63
	168	283	88
	152	148	44
Fried	121	103	44
Veal liver	97	262	76
	70	226	60
	158	158	43
Pork liver	159	196	55
	153	221	69
	122	124	39
Lamb liver	96	238	69
Beef kidney	126	160	32
	81	173	40
	167	223	49
Stewed	112	114	40
Pork kidney	83	177	39
	62	127	27
	137	157	33
Beef heart	143	93	18
	133	121	23
	54	56	12
	166	122	22
Stewed	87	63	19
Veal heart	155	121	25
Pork heart	104	114	25
Pork loin	36	63	20
	74	45	13
	125	68	19
	156	69	21
	162	46	14
	165	45	13
Broiled	37	26	6
Fried	38	41	10
"	93	22	7
Fresh frozen	89	51	17
Veal hindquarter	103	53	14
	134	44	10
	129	72	18
Fried	45	39	—
Lamb leg	80	50	13
Beef spleen	130	63	13
	76	48	—
Stewed	88	42	—
Beef round	105	46	13
	146	51	13
	151	36	—
	154	49	12
	128	29	7
	42	36	10
	157	37	10
	164	28	8
Fried	106	30	—
Broiled	147	34	—
Fried	148	36	—
Pork ham	95	76	19
	32	69	19
	139	45	11
	124	70	17
Fried	141	20	10
"	52	46	—
Roasted	142	27	13
Tenderized	72	21	7
"	119	27	9
"	117	40	14
Smoked	116	58	17
"	102	67	19
"	47	44	14
Smoked, fried	48	44	21
Boiled	115	13	5
Beef pancreas	113	106	28
	64	60	19
Beef brain	144	170	36
Beef lung	78	54	—
	138	72	15
Beef tongue	82	74	20
Light muscle of chicken	65	35	9
Light muscle of chicken	68	27	7
Dark muscle of chicken	66	83	21
Filet of cod	135	21	4
Salmon muscle	136	65	18

[1] Expressed as calcium pantothenate.

DISCUSSION

A critical comparison of the values presented in tables 1 and 2 offers several noteworthy observations. The effect of enzymatic digestion on the pantothenic acid values for liver or kidney is evidently much less than that obtained on muscular tissue. No marked increases in the pantothenic acid values of liver and kidney were observed after pancreatic digestion when compared to the values after clarase and pepsin digestion. Clarase digestion of beef, veal, pork and lamb muscle tissue caused a very definite increase in pantothenic acid content over the values obtained after pepsin digestion of these tissues. In general there was a still further increase in the pantothenic acid content of muscle tissue when the samples were treated with pancreatin. In several samples of heart muscle there was an indication of increased pantothenic acid content both with clarase and pancreatin treatment. The same results were obtained with beef tongue indicating a definite trend toward greater liberation of pantothenic acid in those tissues which contained increased amounts of muscular fiber. However, a sample of beef brain showed more than double the pantothenic acid content after pancreatic digestion when compared to the amount found after pepsin digestion.

The variable effect of the different enzymes on certain of the tissues may furnish some clue as to the form in which pantothenic acid occurs within animal tissues. There are at least two explanations for the apparently greater quantity of "free pantothenic acid" in liver as contrasted with other animal tissues. It would appear likely that in a more highly metabolizing tissue as the liver and kidney, the pantothenic acid occurs free to a greater extent than in structural tissue such as muscle. A second explanation is the probable occurrence of a larger number of enzymes in the liver than in other organs or muscle which would allow increased autolysis. The greater "apparent pantothenic acid" content would thus actually be due to increased amounts liberated from the natural pantothenic acid complexes during preparation. This second explanation finds support in the report by Rohrman

et al. ('34) that fresh liver contains a relatively low pantothenic acid content which increases twelve times upon autolysis. Our drying procedure evidently allowed sufficient autolysis to occur since hot water extraction removed the greater proportion of pantothenic acid in the liver samples. While pepsin digestion gave a marked increase in pantothenic acid content of muscle over that given by simple water extraction, it is probable that a more complete breakdown with pancreatin would give greater liberation of the combined pantothenic acid. Although it would appear that the bacteria could utilize all the free pantothenic acid in the meat preparation, the possibility does exist that the vitamin may be mechanically held within the particles of meat and thus be unavailable to the organism. Any attempts to obtain more complete solution of the tissue would minimize this possibility.

It will be seen that the greatest increases in pantothenic acid were found after pancreatic digestion of cooked meats. For example the sample of stewed heart showed a threefold increase in pantothenic acid content after pancreatic digestion when compared to pepsin digestion. Stewed beef spleen showed twice the vitamin content and several of the commercially treated pork muscle samples showed a two- and threefold increase in vitamin content after the more complete hydrolysis of the sample. The denaturation of the meat proteins may have made the pantothenic acid less extractable after cooking and it was only after pancreatic digestion that the vitamin was completely liberated.

In general, the same relative pantothenic acid potency of the tissues has been found by the microbiological test as by the chick assays. Liver, kidney and heart are outstanding sources of pantothenic acid while muscular and organ tissues such as pancreas, brain, lung, etc., are good sources of the vitamin. The different samples of liver showed some variation in pantothenic acid content but this may be ascribed to the various factors which influence the vitamin content of the carcass such as age of the animal, type of feed consumed, etc.

Such variations within a limited range are also seen in the pantothenic acid values of the muscle tissue.

The loss of pantothenic acid due to household and commercial processing varied somewhat with the type of cooking procedure and with a particular tissue. The pantothenic acid values of stewed beef kidney, stewed beef spleen and stewed beef heart were slightly lower than those found in untreated samples of these tissues. The decrease can be accounted for in large part by the solution of the vitamin in the cooking water. The effect of frying is best shown by comparing parallel samples of fried beef round and untreated beef round from the same cut. Two samples of beef round showed a loss of approximately 30% after frying. Nearly the same loss was observed in the fried pork muscle, but no apparent loss was observed in the fried smoked ham. Several roasted samples showed losses approximating 40%.

Tests on the effect of commercial processing on the stability of pantothenic acid were restricted to pork muscle samples. The tenderizing process caused a loss of 50% of the pantothenic acid while the boiled ham samples showed approximately one-third the original pantothenic acid of the majority of untreated ham samples. Smoking the hams resulted in the least loss of pantothenic acid.

A summary of the vitamin content of meat indicates that liver and kidney are the outstanding sources of nicotinic acid and riboflavin (Waisman and Elvehjem, '41). The results of the pantothenic acid assays showed that liver and kidney also contain high amounts of this vitamin. The muscle tissue and organ tissue are less potent than kidney or liver, yet contain appreciable amounts of pantothenic acid.

SUMMARY

Microbiological assays for pantothenic acid have shown that animal tissues contain considerable amounts of this vitamin. Liver and kidney of the various species were the richest of any of the tissues studied while striated muscle, heart, lung, pancreas, brain and spleen contained appreciable quantities.

It appears that the pantothenic acid in animal tissues occurs in combined form and is liberated most completely by pancreatin digestion. Partial liberation of the vitamin from its complexes was obtained with other enzymes which hydrolyzed the meat proteins less completely. There is a decrease of approximately 30 to 40% of the vitamin in the cooked or commercially processed samples.

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EFFECT OF PECTIN ON THE RETENTION OF DIETARY LEAD (RADIUM D)

HARVEY K. MURER AND LATHAN A. CRANDALL, JR.
Department of Physiology, University of Tennessee College of Medicine, Memphis

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Shields, Mitchell and Ruth ('39) found that inclusion of 32% apple powder in rat diets decreased lead retention by 37% when the food contained 33 p.p.m. of lead as lead arsenate. They point out the insolubility of lead pectate, and suggest that the pectin of the apple may be a principal factor in reducing retention by decreasing the absorbability of this metal. A protective effect of raw apple was reported by Manville et al. ('40), who used even larger amounts of lead arsenate. These authors, however, were able to demonstrate only a better growth of the apple-fed groups (rats, guinea pigs, rabbits). They did not make analyses for lead in the whole animals, but found that bone, kidney, and liver taken from the animals receiving apple might show either more or less lead than the controls.

In order to determine whether pectin itself can decrease the retention of lead, we have carried out experiments on lead retention using purified pectin. We also wished to have the conditions adverse to a demonstration of the possible effect of pectin, and have therefore used a diet high in calcium and with the addition of minimal amounts of lead. Tompsett ('39) presents data which show that the proportion of lead absorbed from the diet decreases with increasing amounts of lead consumed; this effect is most marked on a high calcium diet but is definite on low calcium diets also. The decrease in lead absorption produced by high levels of calcium is greater with

increasing amounts of dietary lead. This effect of calcium on lead absorption has been confirmed by Lederer and Bing ('40). We might therefore expect that other agents which inhibit lead absorption would be less effective when the amount of lead in the diet was in the lower range. By the use of the naturally occurring radioactive lead isotope, radium D, it has been possible to study the effect of pectin on lead absorption when the amounts of lead in the diet are no greater than those present as natural contaminants, since the quantity of radioactive lead which must be added to the diet is negligible in comparison with that already present in most foods. The use of radium D has also made it possible to trace the lead actually absorbed, retained, and excreted, bearing in mind the fact that none of the lead determined by the method could have been present previously in the body of the animal.

PROCEDURE

Radium D (Pb^{210}), which has a half period of 16.5 years, was obtained from disintegrated Radon tubes. The tubes were smashed and the mercury they contained, due to the pumping operation, was removed by vacuum distillation in a micro still, using a fine air stream to carry off the vapors. The active deposit was taken up in a mixture containing 1 part concentrated HNO_3, 1 part concentrated HCl, and 2 parts water. It was then evaporated to dryness, taken up in 1:1 concentrated HNO_3, and diluted so that 25 ml. contained 5 ml. of concentrated HNO_3. This solution also contained traces of the short-lived isotopes of bismuth and polonium, which emit chiefly alpha and beta particles with small amounts of soft gamma rays. It was not necessary to remove these from solution, since their radiation can be easily eliminated by screening the Geiger tube. Various dilutions of this Pb^{210} solution in 1:4 HNO_3 were retained as permanent standards.

A known volume of the Pb^{210} solution was added to 300 gm. of dry starch, the mass vacuum dried, transferred quantitatively to a ball mill, and powdered for 24 hours. Portions of the lead-starch mixture were analysed for radioactivity in

comparison with the permanent standards, and were found to check within 2% of the calculated values. This lead-starch preparation was used in place of a part of the ordinary starch in the diet, the amount added being calculated to supply 1150 "units" of activity per 200 gm. of diet.

The difficulty of determining the weight of lead added to the diet when the only known figure is the approximate radioactivity of the original Radon tubes, is obvious. However, it may be roughly estimated that the amount of lead fed to each rat could not have been more than 8 μg., i.e., the diet would contain not more than 0.04 p.p.m. of radioactive lead. Although the diet was not analysed for total lead, it may be assumed from the data of other investigators on the lead content of common foods that the radioactive lead amounted to only a small fraction of the total lead in the diet. It is certain that the total lead content of the diet was not appreciably higher than that of the usual rat diet.

Four litter mate pairs of rats were used, of which half received the control diet and the others the same diet but with 5% of the ordinary starch replaced by pectin. The compositions of the diets are given in table 1.

TABLE 1

Composition of control and pectin-containing diets.

	CONTROL DIET	PECTIN DIET
	%	%
Whole milk powder	50	50
Starch [1]	39	34
Purified casein	5	5
Dried brewers' yeast	4	4
Sodium chloride	1	1
Osborne-Mendel salt mixture	1	1
Purified pectin [2]	0	5

[1] Consisted of a mixture of ordinary starch with starch to which Pb^{210} had been added, the amount of Pb^{210} starch being the same in each diet.

[2] Citrus products 194 grade, slow set. This was purified by four washings with strongly acidified 60% alcohol followed by one washing with 95% alcohol and air drying.

The animals were kept in all-glass cages and fed from porcelain cups. Urine and feces were caught in glass funnels provided with glass wool for screening and the urine was retained in small flasks. The diets were restricted to prevent large gains in weight, and were mixed with water in the feeding cups to avoid wasting and contamination of the excreta. Each animal was fed 200 gm. of diet which contained 1150 "units" of radioactivity. When this amount had been consumed the animal was etherized, and fecal matter removed from the gastrointestinal tract. Analyses for radioactivity were then carried out separately on the urine and feces for the entire experimental period and on the whole animal.

Preparation of samples for analyses. The usual dry and wet ashing procedures were found tedious and difficult when applied to these samples. We have therefore employed a nitric acid isobutyl alcohol digestion which is simpler and less subject to error. The feces were collected and combined with the contents of the intestine. The glass rat cage was rinsed with 1:1 nitric acid and then with 10% acetic acid and the washings added to the urine sample. The urine, feces, and rat carcass were each placed in a 3 liter beaker and covered with concentrated nitric acid. After frothing had subsided a few milliliters of isobutyl alcohol were added very cautiously while stirring. The violent reaction that results serves to bring the samples into solution very rapidly. In the case of the rat carcass, the fat rises to the surface upon cooling, and is removed with hexane in a separatory funnel, the hexane being washed with cold water. Removal of the fatty acids in this manner aids in further handling of the sample.

The beakers were then placed on asbestos covered low heat hot plates and allowed to evaporate nearly to dryness. The samples were transferred with hot acidified (HNO_3) water to porcelain evaporating dishes, evaporated to dryness on the steam bath and dehydrated in the oven, and finally ashed slowly in the furnace without ignition and below 500° C. With the preliminary treatment, ashing became simple and could easily be accomplished in a few minutes without loss of mate-

rial. About 20 ml. of concentrated HCl were added to the ash and evaporated to dryness on the steam bath. The residue was taken up in hot hydrochloric-citric acid solution containing 100 gm. of citric acid and 100 ml. concentrated HCl per liter, and filtered through a fine sintered glass filter. The filter and evaporating dish were washed successively with hot HCl, hot HCl citric acid solution, and hot 40% ammonium acetate. A few pellets of NaOH were then added to the evaporating dish, dissolved in a few milliliters of water with rotation to insure complete wetting of the dish, and this solution evaporated to dryness. The residue was taken up with hot water and this was poured directly into the filtrate. The dish was finally rinsed with hot 1:4 HCl. The combined filtrate and washings were cooled, 20 ml. of 50% citric acid solution were added, and the pH was adjusted to 3.0–3.4 with concentrated ammonia using bromophenol blue as an outside indicator. Five milligrams each of copper sulfate and lead nitrate were added to the solution, and the heavy metals were precipitated by a stream of H_2S. The solution was filtered immediately on a fine sintered glass filter, and the sulfides remaining in the beaker and on the filter were dissolved with 5 ml. of hot concentrated nitric acid. The nitric acid filtrate containing the dissolved lead was caught in a 25 ml. volumetric flask, cooled, and made to volume. Samples of 5 ml. of the solutions thus prepared were placed in flat bottomed 1.5×4 cm. vials, and sealed. The extra lead and copper added before saturation with H_2S give an easily workable precipitate and insure that the traces of radioactive lead will be quantitatively recovered.

For the determination of radioactivity a Geiger-Mueller apparatus was constructed using the quenching circuit described by Johnson ('38). This was modified to include both the grid potential control as suggested by Coven ('39) and the recording circuit of Neher ('39). The regulated high voltage supply was adapted from that used by Lifschutz ('39). The Geiger-Mueller tube was mounted in a bakelite box with a hole drilled above the tube so that vials containing the radioactive solutions could be placed in exactly the same position

for each determination. By keeping the position of vial and volume of solution constant, the solid angle was the same for each determination. The procedure was such that the concentration of reagents was the same in all of the unknown samples and in the standard Pb^{210} solutions, thus controlling the absorption factor. Counts on the unknown solutions were made alternately with counts on the two standard solutions most nearly equal to the unknown in activity. The average of four 1-hour counts on the unknown was used, and was converted into milliliters of original Pb^{210} solution from a curve plotted with milliliters of original solution as the abscissa and counts per minute as the ordinate.

RESULTS AND DISCUSSION

Table 2 compares the lead retained and excreted via urine and feces in the four litter mate pairs with and without pectin. Since by the procedure used all of the lead should be recoverable, and since contamination with ordinary lead is of no consequence, it has been possible to obtain exceptionally good recoveries of the dietary lead.

The retention of dietary lead, which for our control group averaged 15.8%, may be compared with retentions observed by others who have administered larger amounts. Thus Tompsett ('39) found 28% retained by mice given 0.7 mg. over a 14-day period, when the animals received a high calcium diet. Shields, Mitchell, and Ruth ('39), who fed much larger amounts over longer periods, report 1.0 to 1.5% retention. Kehoe et al. ('40) state that retention is negligible in man when somewhat more than 1 mg. per day is taken over a 3½ year period. The proportion of lead retained is evidently in inverse proportion to both dosage and length of time over which a given daily dosage is administered. The retention of about 16% in our experiments is in line with previous observations from short term, low dosage experiments.

The distribution between urine and feces, as determined by us, is probably somewhat inaccurate since under the conditions of our experiments some leaching of the feces by urine

TABLE 2

Effect of pectin on the retention and excretion of lead (radium D) in the diet of the rat.

DATE GIVEN	INITIAL WEIGHT	FINAL WEIGHT	TOTAL LEAD UNITS FED	TOTAL LEAD UNITS RECOVERED			PER CENT RECOVERED	PERCENTAGE DISTRIBUTION		
				Animal	Urine	Feces		Animal	Urine	Feces
	gm.	*gm.*								
1 Control	152	151	1150	175	120	850	99.5	15.2	10.4	74.0
2 Pectin	153	148	1150	150	87	925	101.5	13.0	7.6	80.4
3 Control	142	159	1150	187	112	825	97.7	16.2	9.7	71.6
4 Pectin	140	148	1150	127	87	913	98.0	10.0	7.6	79.4
5 Control	112	140	1150	185	150	800	98.5	16.1	13.0	69.5
6 Pectin	112	142	1150	154	112	900	101.5	13.4	9.7	78.2
7 Control	152	156	1150	180	122	825	98.0	15.6	10.6	71.7
8 Pectin	152	154	1150	125	75	935	98.5	10.9	6.5	81.2
Means of controls								15.8	10.9	71.7
Means of pectin-fed								11.8	7.9	79.8
Differences of means and standard errors of differences								4.0 ± 0.84	3.1 ± 0.97	8.1 ± 1.11
							t	4.8	3.1	7.3
							P	0.01	0.02	0.001

Statistical analysis of percentage distribution by small sample method (Fisher). Note that figures in per cent can be treated as absolute values, since they are parts of the constant amount fed to each rat.

could have occurred. The 72% of the Pb^{210} eliminated in the feces of the control group is therefore a minimum figure, and the urine values may be slightly too high.

Our results reveal a clear-cut and statistically significant effect of pectin on lead absorption and retention. The amount of lead retained in the body at the end of the experiment is, on the average, 24% less in the pectin-fed rats than in the controls. The difference between the average body lead in control and pectin rats is 4.0 and its standard error ± 0.84. The probability (Fisher's table of t) of this difference occurring by chance is less than 1 in 100. The smaller urinary output of lead and the greater fecal excretion in the pectin-fed animals as compared with the controls are likewise statistically significant, P being 0.02 and 0.001 respectively. In order to interpret the greater fecal excretion and smaller body and urine content of the pectin-fed animals as evidence of diminished absorption, it is of course necessary to neglect the possibility of absorption and re-excretion via the digestive tract. However, we know of no reason why the administration of pectin should increase biliary or intestinal lead secretion. On the other hand, an inhibition of lead absorption by pectin is readily understandable. Not only pectic acid, but pectin itself forms insoluble lead compounds. Kertesz ('40) has shown that pectin passes unchanged through the small intestine, and we have confirmed his findings in this laboratory (unpublished). The formation of an insoluble lead-pectin complex would therefore be expected to result in the transmission of increased amounts of lead through the small bowel, and although pectin may be broken down in the large intestine by bacterial fermentation there would be relatively little absorption of the liberated lead in this region. The addition of 5% pectin to the diet does not cause diarrhea and we have no reason to believe that the diminished absorption of lead can be accounted for by an increased rate of passage through the small intestine.

Finally, it should be emphasized (1) that the total amount of lead in the diet used by us is that which may be found in the average diet of rat or man, (2) that the diet was rich in

calcium, and (3) that if the effect of pectin on lead absorption is analogous to that of calcium one would anticipate more marked effects with increasing concentrations of dietary lead. This indicates that the 24% reduction in lead retention which we have observed is likely to be exceeded under less unfavorable conditions.

SUMMARY

Rats were fed 200 gm. each of a diet containing not more than 0.04 p.p.m. of Pb^{210}. One group served as controls, another received 5% pectin which replaced an equal amount of starch.

The control group retained 15.8% of the radioactive lead, and excreted 10.9% in the urine and 71.7% in the feces. The pectin-fed animals retained an average of 24% less lead than the controls. Significantly less was eliminated in the urine and significantly more in the feces.

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VITAMIN SUPPLEMENTATION OF U. S. ARMY RATIONS IN RELATION TO FATIGUE AND THE ABILITY TO DO MUSCULAR WORK [1]

ANCEL KEYS AND AUSTIN F. HENSCHEL

Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis

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The recognition of borderline vitamin deficiency states prompts the suggestion that a direct relation between the intake of certain vitamins and physical vigor may extend even above the subclinical deficiencies. During the past several years the popular belief has grown that a superabundance of these vitamins may produce extraordinary stamina or at least may be helpful in meeting physical or mental strain.

These possibilities were of such obvious importance as to merit careful investigation. This paper is a report on such studies made under rigidly controlled conditions on personnel of the U. S. Army.

EXPERIMENTAL PROGRAM

The general plan was to study the physiological and biochemical responses to standard exercise in representative volunteers. Four main experimental series were carried out on twenty-six soldiers, involving a total of 256 experiments.

[1] This work was sponsored by the National Research Council, Division of Medical Sciences and was specifically approved by the Subcommittee on Clinical Investigation and the Subcommittee on Nutrition. The use of soldiers as subjects was authorized by the Secretary of War, acting on the recommendation of the Surgeon-General, U. S. Army. Special financial assistance was also provided by the Mayo Foundation and by the Department of Athletics, University of Minnesota. Important aid was rendered by the Works Progress Administration under the University of Minnesota Project No. 8760, Sub-project No. 380.

Throughout the entire period of study all the men subsisted on standard Army Post rations; during part of this time they received daily supplements of vitamins and during the remainder they received placebos identical in taste and appearance to the vitamin capsules.

In the first experimental series each man reported at the laboratory twice a week for a half-day experiment. In the succeeding experimental series each man reported once a week for a 24-hour experiment. In all cases the experiments consisted of physiological and biochemical observations in a standard state of rest, a standard period and intensity of muscular exertion, followed by physiological and biochemical observations at successive stages of recovery. During the actual exercise the pulse rate was continuously recorded.

TABLE 1

Duration of exercise in minutes and the average external work accomplished per experiment, in kilogram meters, for the several experimental series.

TIME	SERIES I		SERIES II		SERIES III		SERIES IV	
	Time	Kg. M.	Time	Kg. M.	Time	Kg. M.	Time	Kg. M.
Afternoon	15	14,490	90	55,150	90	57,280	90	57,360
Morning	—	———	120	73,430	90	57,280	90	57,360

The exercise consisted in marching for a definite period on the motor-driven treadmill at a constant speed of 3.85 miles per hour and a 12.5% angle of climb in the first series and 10% in all subsequent series. Standard packs were carried and the clothing, time of day, relation to previous meals and exertion were standardized. The exercise room was maintained at 78° F., with relative humidity between 40 and 60% saturation. The exercise was severe enough to be somewhat beyond the capacity of the average untrained college student. The duration of exercise and external work accomplished are shown in table 1.

In order to eliminate training effects a preliminary period of 2 to 4 weeks in each experimental series was devoted to

training. To ensure that training and other extraneous effects would be at a minimum the men in each experimental series were placed in two groups, one of which received the vitamin supplements during the first half of the experimental period proper while the other group received the vitamins in the second half of the period. The supplements used are shown in table 2.

TABLE 2

The daily vitamin supplements, in mg. The daily administration of the capsules was checked at the Fort Snelling Dispensary.

	THIAMINE Cl (B_1)	NICOTINIC ACID AMIDE	CALCIUM PANTOTHENATE	RIBOFLAVIN (B_2)	PYRIDOXINE (B_6)	ASCORBIC ACID (C)
Series 1	5	100	—	—	—	100
Series 2	7	100	20	10	10	200
Series 3	17	100	20	10	10	200
Series 4	—	—	—	—	100	—

The physiological and biochemical variables, which were measured to assess the results of the exercise and the effects of the vitamins on them, included: the pulse rate, heart size, stroke output of the heart, oxygen consumption, respiratory quotient, urinary nitrogen and ketone body excretion, and concentration in the blood of lactate, sugar hemoglobin and ketone bodies.[2] In experimental series III and IV total urinary excretion of ketone bodies and of nitrogen was measured and 2-hour sugar tolerance curves were obtained at the end of the exercise.

[2] The following methods were used: heart rate — integrating cardiotachometer; heart size and stroke output — roentgenkymographic method of Keys and Friedell ('39), and Keys et al. ('40); oxygen consumption and respiratory quotient — collection and measurement of expired air in a compensated gasometer and direct Haldane analysis of the gas samples; blood hemoglobin — acid hematin method with the Evelyn photo-electric colorimeter; blood sugar — Folin-Wu modification of Benedict's method with the Evelyn colorimeter (Folin-Wu, '20; Folin, '29); blood lactate — Edward's modification of the method of Friedeman, Cotonio and Schaeffer (Edwards, '38); blood acetone and other ketone bodies — method of Barnes and Wick ('39); blood bisulfite binding substances — method of Schrader ('40); urinary nitrogen — direct micro-Kjeldahl method; urinary acetone and other ketone bodies — method of Van Slyke ('17, '29).

RESULTS

The detailed results of the several experimental series are too extensive for publication here. Grand averages for some of the more significant variables are given in tables 3 and 4.

Only one man reported a marked change in his ability to carry out the heavy exercise but his subjective improvement occurred during his period on the placebos. Other subjective reports of slight improvement occurred equally in placebo and vitamin supplement periods.

The objective measurements showed that the vitamin supplementation had no effect either on the stroke output of the heart or on the heart rate before, during or after exercise. The heart size was slightly reduced, on the average, after the heavy work as compared with the size in previous rest, but this small change was shown in both placebo and supplement periods.

The average concentration of lactic acid in the blood in series I was increased almost exactly threefold by the exercise but this was unaffected by the vitamin supercharging. The rate of removal of lactic acid from the blood in recovery was likewise independent of the vitamin supplementation. In all the other experimental series the blood lactate level remained relatively constant before and after the work period both on placebos and on vitamin supplements.

The bisulfite binding substances in the blood were measured before and after work in the first experimental series but no change was found in either placebo or vitamin supplement periods.

The blood sugar was decreased by the work in all the experiments by average amounts of 14.1 to 18.4 mg. per 100 cc. but this decrease was not different in the vitamin supplement periods as compared with the placebo periods. Blood sugar measurements in series II and III showed, on the average, a somewhat more rapid return of blood sugar toward normal values during recovery in the vitamin supplement periods but the difference was not statistically certain. In series I, in

TABLE 3

Grand averages for some significant variables.

EXPERIMENTAL SERIES I

Blood Lactic Acid, mg. per 100 cc.

Period on	Rest	After Work 2 min.	After Work 10 min.	K [1]
Vit.	11.6	33.2	24.2	0.105
Plac.	10.6	33.2	23.0	0.089

Heart Rate, Beats per min.

Period on	Rest Seated	1st 30 sec. Recovery	9th min. Recovery
Vit.	82.5	147.6	103.2
Plac.	81.5	148.1	106.0

Blood Sugar, mg. per 100 cc.

Period on	Rest	After Work 2 min.	After Work 10 min.
Vit.	101.0	86.0	84.8
Plac.	96.9	80.8	85.1

Heart Size and Stroke

Size in sq. cm. frontal projection area in systole, stroke in cc. net systemic output.

Period on	Rest Size	Rest Stroke	3 min. after work Size	3 min. after work Stroke
Vit.	105.7	63.6	104.4	62.9
Plac.	107.3	63.6	104.5	62.6

[1] $K = \frac{2.303}{\Delta t} (\log_{10} C_1 - \log_{10} C_2)$,

where Δt is time in minutes between recovery blood samples, and C_1 and C_2 are concentrations in the 2 recovery blood samples.

EXPERIMENTAL SERIES II

Blood Lactic Acid, mg. per 100 cc.

Rest	End of Work	10 min. Recovery
8.53	12.16	11.33
8.76	10.85	10.39

Heart Rate, Beats per min.

Rest Seated	Last min. Work	2nd min. Recovery
63.6	150.4	117.7
67.1	150.9	114.2

Blood Sugar, mg. per 100 cc.

Rest	End of Work	10 min. Recovery
90.3	72.9	82.5
94.5	76.1	86.2

Blood Hemoglobin, gm. per 100 cc.

Period on	Rest	End of Work	10 min. Recovery
Vit.	13.9	14.9	14.2
Plac.	14.0	15.0	14.4

Dextrose Tolerance, mg. per 100 cc.

Period on	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.
Vit.	90.3	161.6	158.8	150.6	140.5
Plac.	94.5	164.1	162.5	161.7	135.7
No work	92.1	128.7	108.8	95.8	78.4

Oxygen Consumption and R.Q.

O_2 consumption in cc. at S. T. P. per min.

Period on	Rest before work O_2	Rest before work R. Q.	10–20 min. after work O_2	10–20 min. after work R. Q.	25–35 min. after work O_2	25–35 min. after work R. Q.
Vit.	263.9	0.845	313.8	0.794	278.2	0.753
Plac.	263.9	0.849	309.0	0.764	291.5	0.770

TABLE 4

Grand averages for some significant variables.

EXPERIMENTAL SERIES III

EXPERIMENTAL SERIES IV

Blood Sugar, mg. per 100 cc.

Period on	Rest	End of Work	10 min. Recovery	Rest	End of Work	10 min. Recovery
Vit.	93.6	79.5	86.1	92.6	81.3	85.1
Plac.	94.2	80.1	86.6	85.7	79.3	82.7

Dextrose Tolerance, mg. per 100 cc.

Period on	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.	Rest	0.5 hr.	1 hr.	1.5 hr.	2 hr.
Vit.	93.6	140.1	151.8	163.0	136.1	92.6	132.4	146.8	129.6	107.5
Plac.	94.2	133.8	149.6	155.3	128.8	85.7	130.5	150.4	137.1	129.3

Pulse Rates, Beats per minute

Period on	Rest Standing	Last min. Work	2nd 60 sec. Recovery	Rest Standing	Last min. Work	2nd 60 sec. Recovery
Vit.	90.6	143.1	102.4	77	136	92.5
Plac.	88.0	146.4	104.2	75	135.5	93.5

Urinary Nitrogen and Ketone Bodies

Total elimination in 14 hours, from 10 P. M. to noon, including morning work. Nitrogen in gm., ketone bodies as acetone in mg.

Period on	Nitrogen	Ketone bodies	Nitrogen	Ketone bodies
Vit.	5.84	36.3	7.6	37.4
Plac.	5.67	37.7	5.4	34.3

Oxygen Consumption and R. Q.

O_2 consumption in cc. at S. T. P. per min.

Period on	Rest before work		10–20 min. after work		25–35 min. after work	
	O_2	R. Q.	O_2	R. Q.	O_2	R. Q.
Vit.	243.1	0.811	290.4	0.795	263.1	0.771
Plac.	242.0	0.813	304.3	0.791	253.3	0.761

Blood Acetone, mg. per 100 cc.

Period on	10 min. after work	0.5 hr. after work	1.5 hr. after work
Vit.	4.5	4.2	1.9
Plac.	3.7	3.6	2.7

which the severe exercise lasted only 15 minutes, the reverse was observed on the average, but the difference was small.

In all experiments the hemoglobin concentration in the blood rose as a result of the exercise (average 7.1%). This hemoconcentration was independent of vitamin supplementation. The rate of oxygen consumption in rest before work and in recovery was not appreciably affected by the vitamin supplementation.

There was no apparent effect of the vitamin supplement on the respiratory quotient in these experiments although the R.Q. declined to values averaging below 0.80 in series II and III.

Sugar tolerance tests made at the end of work showed a pronounced departure from the normal results obtained on the same men on control days without work. The severe work produced a metabolic state resembling mild diabetes mellitus with some ketosis. However, there was no difference qualitatively or quantitatively in the appearance of these phenomena during either the placebo or vitamin supplemented periods.

The total elimination in the urine of nitrogen and of ketone bodies was measured in series III and IV. The average results were almost identical in the periods on vitamin supplementation and on placebos.

DISCUSSION

In 1934 Baëna injected normal rats with a mixture of ascorbic acid and adrenal cortical extract and reported an increased endurance to muscular strain which was not evident when the cortical extract was used alone. Brack ('36) reported improved muscular performance with ascorbic acid plus cortical extract in isolated muscles but found that the ascorbic acid itself was without effect. Wachholder and Podesta ('37), Guarnaschelli-Raggio ('38), and Sievers ('39) reported somewhat discordant results on the working capacity of isolated frog muscles bathed in ascorbic acid solutions.

The reports on the action of excess thiamine chloride are likewise difficult to interpret. Minz and Agid ('37) and Hano ('37) stated that the working capacity of muscle is improved by injections of thiamine. Briem ('39) claimed that the addition of thiamine to the medium bathing isolated muscles increased the number of contractions induced by acetylcholine. Kaiser ('39) denied this and explained Briem's results on the basis of neglect of control of the acidity of the medium.

In the course of studies in induced thiamine deficiency Williams, Mason, Wilder and Smith ('40) obtained some impressions that an unusually large intake of thiamine may promote a degree of physical vigor not seen on an ordinary intake of the vitamin. Gounelle ('40) gave 10 to 15 mg. of thiamine chloride daily to cyclists and football players with results in competition that were considered favorable.

Morell ('40) reported improved endurance in bicycle tests with two men who received 100 gm. of a vitamin mixture as they were approaching exhaustion. The mixture contained, in each 100 gm., 62 gm. dextrose, 100 mg. nicotinic acid, 11.7 mg. thiamine chloride, and 200 mg. ascorbic acid. These results are readily explained by the additional supply of carbohydrate fuel.

Analysis of the garrison rations supplied at Fort Snelling during the 10 months of the present study was made on the basis of the stock purchase, consumption records and daily menus. The grand average daily intake per man on these garrison rations was 1.7 mg. of thiamine chloride, 2.4 mg. of riboflavin, and 70 mg. of ascorbic acid. The actual food consumption records are on file in this laboratory.

Vague statements in the literature to the effect that vitamin supplementation of "adequate" diets may promote increased well-being and vigor need not be discussed. Sherman and Campbell ('37) offer experimental data on rats. Actually, however, the latter results only show that a laboratory diet which allows reproduction and freedom from nutritional disease does not necessarily promote maximal growth.

SUMMARY

Soldiers maintained on standard U. S. Army garrison rations were repeatedly subjected to standardized severe exercise on the motor-driven treadmill. Circulatory, metabolic and blood chemical responses were measured.

In four series of studies involving 256 experiments, large daily supplements of thiamine chloride, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and ascorbic acid were administered over periods of 4 to 6 weeks alternating with equal periods of placebo administration.

In neither brief extreme exercise nor in prolonged severe exercise and semi-starvation were there indications of any effects, favorable or otherwise, of the vitamin supplementation on muscular ability, endurance, resistance to fatigue or recovery from exertion.

Healthy young men expending an average of 3700 to 4200 calories per day are not benefited by a daily supply of more than 1.7 mg. thiamine chloride, 2.4 mg. riboflavin, and 70 mg. of ascorbic acid.

It is concluded that no useful purpose would be served by enrichment of present U. S. Army garrison rations with the vitamins studied.

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SERUM PHOSPHATASE IN EXPERIMENTAL SCURVY

HARRY SHWACHMAN AND BERNARD S. GOULD

Department of Pediatrics, Harvard Medical School, Infants' and The Children's Hospitals, Boston, and Laboratories of Biochemistry, Department of Biology and Public Health, Massachusetts Institute of Technology

TWO FIGURES

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INTRODUCTION

The relationship between phosphatase activity and ascorbic acid has been the subject of considerable investigation. It has been adequately demonstrated that ascorbic acid does not activate alkaline phosphatase in vitro (King and Delory, '38; Thannhauser et al., '37, '38; Albers, '40). However, in vivo experiments with guinea pigs (Scoz et al., '37; Todhunter and Brewer, '40) indicate that the withdrawal of ascorbic acid from the diet results in a lowering of serum alkaline phosphatase activity. In infantile scurvy the serum phosphatase is reduced, and rises after the administration of ascorbic acid (Shwachman, '41).

The present investigation has been undertaken to determine further the relationship in vivo between serum phosphatase activity and scurvy with a view to elucidating the more exact role of ascorbic acid. The activity of serum phosphatase was determined during the development of scurvy, when manifest symptoms of the disease were present, and during treatment with varying amounts of ascorbic acid. Estimations were made of the serum "acid" phosphatase levels of normal and scorbutic animals since no data have been reported on the possible variation in scurvy of other phosphatases than the "alkaline."

METHODS

Guinea pigs [1] 6 to 7 weeks of age and weighing from 250–275 gm. were separated into groups of two or three and housed in clean wire cages. The animals were fed a scorbutogenic diet consisting of equal parts of skim milk (heated at 90°C. for 12 hours), rolled oats and bran fed ad libitum. The diet was supplemented by 1 cc. of cod liver oil every 4 to 5 days. Certain animals were not given any ascorbic acid. To others, a measured amount of ascorbic acid dissolved in water immediately before use was fed by dropper. Animals fed the scorbutogenic diet, but no ascorbic acid, invariably showed clinical evidence of scurvy in 18–24 days. At autopsy, extensive hemorrhages and fragility of the bones were observed. Histological and roentgenographic [2] confirmation of scurvy was obtained. When supplemented with ascorbic acid the scorbutogenic diet was adequate for normal growth of the animals as was evident from the increase in weight and absence of symptomatology.

Serum "alkaline" phosphatase [3] *determination.* The animals were bled directly from the heart under light ether anesthesia. Approximately 2.5 cc. of blood were withdrawn for each determination. Todhunter and Brewer ('40) have demonstrated that ether anesthesia under these conditions does not affect the serum phosphatase activity.

Serum "alkaline" phosphatase was estimated by a modification of the Bodansky method (Shwachman, '41) using 0.5 cc. of fresh serum incubated with sodium β-glycerophosphate [4] in veronal buffer at pH 8.6. In addition, $Mg.^{++}$ was added to a final concentration of 0.009 M. The reaction was carried out at 37°C. for 60 minutes. Acid-soluble inorganic phosphate

[1] These animals were made available to us by Mr. E. J. Staff of the Rhode Island Department of Health to whom we express our thanks.

[2] We are indebted to Drs. Sidney Farber, Nathan Rudo, and Theodore Ingalls for the histological and roentgenographical examinations.

[3] "Alkaline" phosphatase, as used in this report, refers to the enzyme whose optimal activity is in the pH range 8.5–9.0. "Acid" phosphatase refers to the enzyme with pH optimal activity at 4.9–5.0.

[4] Obtained from the Eastman Kodak Company.

was determined by the method of Fiske and Subbarow ('25) using a photoelectric colorimeter. The activity is expressed in units, each equivalent to 1 mg. of inorganic phosphorus liberated per 100 cc. of serum under the conditions of the experiment.

Serum "acid" phosphatase. Serum "acid" phosphatase was estimated by the method of Gutman and Gutman ('40) using sodium β-glycerophosphate (0.5%) as the substrate in veronal (0.212%) — acetate buffer at pH 5.0. No activation by $Mg.^{++}$ of the acid phosphate was observed and, therefore, was omitted from the substrate. Incubation was at 37°C. for 3 hours. Since we have confirmed the observation that the rate of glycerophosphate hydrolysis is linear over the 3-hour period, the values are given in units corresponding to the mg. inorganic phosphorus liberated in 1 hour per 100 cc. serum.

EXPERIMENTAL

Serum "alkaline" phosphatase in scurvy. Thirty 6-week-old animals were divided into groups of six animals each and fed the scorbutogenic diet. The diet of one group was supplemented by 1 mg. ascorbic acid daily; a second group received 6 mg. ascorbic acid daily; two groups received no added ascorbic acid. One group was fed a diet of prepared soy meal [5] chow supplemented with fresh vegetables. Estimations of serum phosphatase of half the number of each group were made approximately every 5 days so that each animal was bled once in about 10 days. Near the end of the experiment estimations were carried out more frequently. The results are represented in figure 1.

During the first 5 days there is a marked drop in the "alkaline" phosphatase activity of all the animals except those on the vegetable diet. This drop appears to be due to the refusal of the animals to eat the scorbutogenic diet even when supplemented with large doses of ascorbic acid. Subsequently, the serum alkaline phosphatase of the animals

[5] Purina.

receiving ascorbic acid rises, and then declines slowly with increase in age of the animals. The animals receiving no supplement of ascorbic acid show a continued marked decline in serum alkaline phosphatase to a low level of about 2 units. In one instance the level fell to as low as 0.3 units (fig. 2). In contrast, animals fed the scorbutogenic diet showed no variation in serum acid phosphatase from those on an adequate diet.

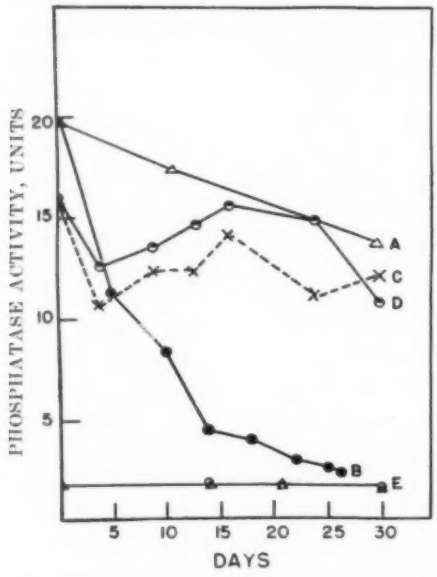

Fig. 1 The serum "alkaline" and "acid" phosphatase activity of normal and scorbutic guinea pigs. Alkaline phosphatase values of guinea pigs fed: A, vegetable diet; B, scorbutogenic diet; C, scorbutogenic diet supplemented with 1 mg. ascorbic acid; D, scorbutogenic diet supplemented with 6 mg. ascorbic acid; E, "acid" phosphatase values of guinea pigs fed vegetable diet, △ and scorbutogenic diet, 0.

Animals fed a fresh vegetable diet supplemented by large doses of ascorbic acid showed no significant differences in phosphatase levels from animals receiving the same diet without an ascorbic acid supplement.

The animals which received the scorbutogenic diet supplemented by 1 mg. of ascorbic acid in most cases maintained almost the same alkaline phosphatase level as the animals receiving 6 mg. daily, although two animals receiving the 1 mg. supplement daily developed mild scurvy (phosphatase values, 3.2 and 4.2 respectively). This indicates that 1 mg. of ascorbic acid daily is a critical dose and that a larger supplement of ascorbic acid is required to maintain the phosphatase level and to protect the animal from scurvy. That 1 mg. of ascorbic acid given daily is a critical dose was also apparent from the change in weight of these animals. The average weight of the animals receiving a supplement of 1 mg. ascorbic acid daily was 285 gm. at the end of 35 days, or an average gain of 17 gm. On the other hand, the final weight of the animals receiving 6 mg. daily was 351 gm. or an average gain of 73 gm. The average loss in weight of the animals on the unsupplemented diet was 59 gm. during the same period. However, in most animals a dose of 1 mg. ascorbic acid daily was as effective as the excessive dose in maintaining the phosphatase at a normal level.

Four 500-gm. guinea pigs were fed the scorbutogenic diet. After 1 month on the diet the serum phosphatase fell from 12.8 units to 2.0 units. This would indicate that the decrease in phosphatase during scurvy is not characteristic of very young animals only.

We have been unable to confirm the observation of Scoz et al. ('37) that there is an initial rise in serum alkaline phosphatase after the withdrawal of vitamin C from the diet, which is then followed by a decline in serum phosphatase. The present data indicate no increases in phosphatase activity, either permanent or transitory at any stage during the development of scurvy.

Effect of "scorbutogenic diet" [6] *on serum alkaline phosphatase of animals (rabbits) which do not develop scurvy.* Bodansky ('34) has demonstrated that modifications of diet

[6] Scorbutogenic diet refers to the diet which, when fed to guinea pigs, produces scurvy.

may induce changes in the serum alkaline phosphatase activity. Although it has been shown that the serum alkaline phosphatase activity of scorbutic animals is restored to normal levels solely by the administration of ascorbic acid, it was deemed desirable to determine whether the scorbutogenic diet itself induced any changes in the serum alkaline phosphatase activity of an animal that is able to synthesize ascorbic acid and does not develop scurvy. Two litters of young rabbits, one litter 31 days old and the other 50 days old, were divided and half the number in each litter placed on

TABLE 1

The effect on the serum phosphatase activity of feeding an ascorbic acid deficient diet to young rabbits.

RABBITS	INITIAL AGE	INITIAL SERUM PHOSPHATASE	ON DIET	FINAL SERUM PHOSPHATASE
	weeks	*units*	*weeks*	*units*
N1-a[1]	4½	17.1	4½	9.6
N1-b	4½	14.7	4½	10.7
S1-a	4½	17.8	4½	11.4
S1-b	4½	15.7	4½	13.2
N2-a	7	7.0	3½	6.3
N2-b	7	9.6	3½	6.9
S2-a	7	9.2	3½	8.5
S2-b	7	9.7	3½	6.0

[1] N: refers to an animal on a diet consisting of purina rabbit chow supplement with fresh green vegetables.

S: refers to animals fed the ascorbic acid deficient diet described previously. Numeral indicates litter.

the "scorbutogenic" diet while the other half was fed a diet of chow and fresh vegetables. The animals on the "scorbutogenic" diet gained very little weight as compared with those on the commercial food. The serum phosphatase values determined after 3½ and 4½ weeks (table 1) indicate that the "scorbutogenic" diet in itself has no apparent effect on serum alkaline phosphatase.

Evidence that low serum phosphatase activity in scurvy is due to absence of the enzyme. The question arises as to whether the low phosphatase activity in severe scurvy is due

to the absence of some activator essential for phosphatase activity, to the presence of some inhibitor, or to a quantitative alteration in the enzyme.

If the lowering of serum phosphatase of scorbutic animals and the rise after treatment with ascorbic acid were due to an alteration in activator or inhibitor concentration, the addition of a high activity serum to a low activity serum would result in a disproportionate change in resultant activity. If the low activity is due to the presence of an inhibitor the mixture of low and high activity sera should lead to a disproportionate lowering of activity. If activity is dependent on the concentration of enzyme the resultant activity should equal the sum of the separate activities. Experiments were carried out using mixtures of extremely low activity sera (scorbutic) and high activity sera (young normal) made up of varying proportions of each. No activation of the phosphatase in low activity sera by high activity sera or inhibition of high activity sera by those of low activity could be demonstrated. The observed activity is essentially equal to the sum of the activity calculated from the activity of each. It appears, therefore, that the change in scurvy involves an actual decrease in the enzyme.

Effect of administration of ascorbic acid on serum alkaline phosphatase of scorbutic guinea pigs. Fifty-five animals were fed the scorbutogenic diet for 18–20 days, after which time practically all showed clinical signs of scurvy. Serum phosphatase estimations were made and the animals were then fed the scorbutogenic diet supplemented with varying amounts of ascorbic acid daily. Estimations of the serum alkaline phosphatase were carried out periodically. The results shown in figure 2 represent the response for individual animals. Most striking is the prompt increase in serum phosphatase after the administration of ascorbic acid and the decrease which follows the withdrawal of ascorbic acid. This is best illustrated by animals 3, 4, 10A and 15A (fig. 2). The speed of the response is illustrated by animals S10 and S12 where, 24 hours after administration of ascorbic acid, a small increase

is apparent, and after 48 or 72 hours the increase is more marked. An examination of the data of animal 10A (fig. 2) which was given a minimal amount of ascorbic acid indicates that the rise is slow but eventually as great as that obtained with large doses in other scorbutic animals. If the supple-

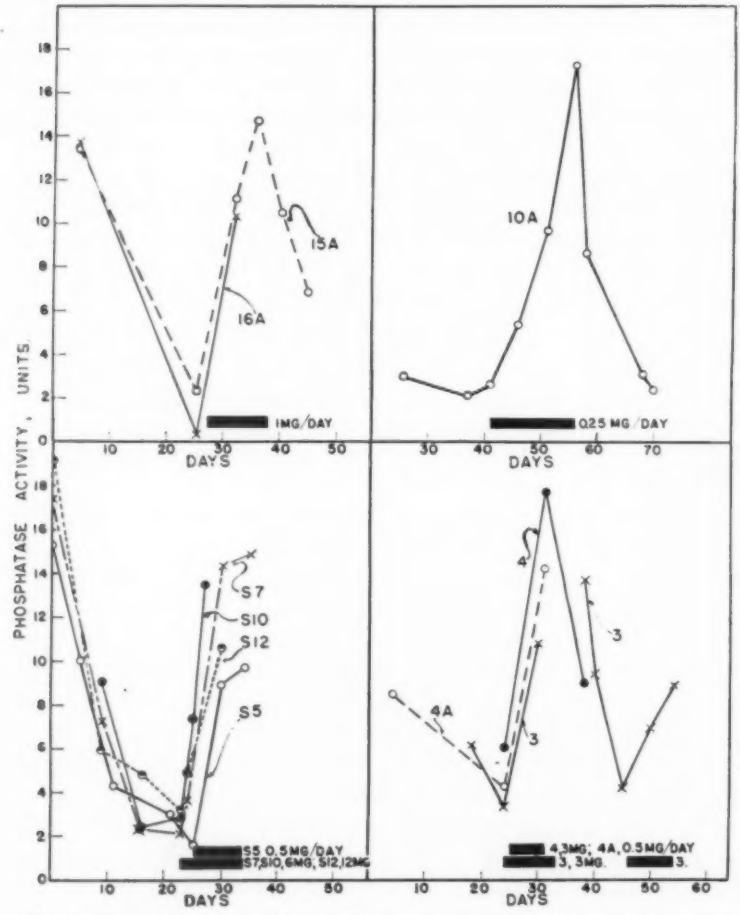

Fig. 2 The effect of ascorbic acid on the serum alkaline phosphatase in scorbutic guinea pigs.

ment of ascorbic acid is increased to 0.5 mg. daily (S5 and 4A) the rise becomes immediate. The fall in phosphatase activity is equally prompt when ascorbic acid is withdrawn from the diet. An examination of figure 2 shows clearly that the increase in phosphatase activity is practically independent of the dosage. These observations would suggest that the increase in phosphatase activity is not due to a direct activation of the enzyme by ascorbic acid in the circulating blood but rather to the stimulation of phosphatase-producing centers (Gould and Shwachman, '42). This rapid change is probably a reflection of the histological response which has been shown by Wolbach and Howe ('26) to be definite in 24–48 hours. More direct evidence to support this view is our failure to obtain activation of the low serum phosphatase from scorbutic animals with ascorbic acid in vitro.

It is noteworthy that large doses of ascorbic acid administered to normal animals have no appreciable effect in raising the phosphatase level, whereas small doses administered to scorbutic animals will result in a marked increase in the serum phosphatase activity. Often the resulting response may be higher than the normal level. This has likewise been observed in infantile scurvy (Shwachman, '41).

In the course of the present study no scorbutic animal failed to show either an increase in serum phosphatase after the administration of ascorbic acid or a decline after withdrawal of the vitamin.

"Acid" phosphatase. Twenty guinea pigs, consisting of sixteen 6 weeks old and four adult 500-gm. animals, were divided into groups some of which were fed a diet of chow and fresh green vegetables, and others were given the scorbutogenic diet. At intervals the "alkaline" and "acid" serum phosphatase activities on the samples were determined. The results (fig. 1) indicate quite clearly that while there is a pronounced decline in the "alkaline" phosphatase activity of the sera of scorbutic animals, there is no change in the activity of the "acid" phosphatase. Moreover, while there is a regular decline in the serum "alkaline" phosphatase ac-

tivity of normal animals with increase in age, there appears to be no corresponding decline in the "acid" phosphatase activity.

Blood acid soluble inorganic phosphate. The method employed for the estimation of phosphatase activity involves in every analysis a control for the inorganic phosphate of the serum. The values obtained for normal and scorbutic animals have been compared and no significant difference could be established.

SUMMARY AND CONCLUSIONS

1. Guinea pigs fed a scorbutogenic diet show an immediate and continued decline in "alkaline" serum phosphatase activity reaching extremely low levels.

2. Animals (rabbits) able to synthesize ascorbic acid show no decline in serum alkaline phosphatase when fed a scorbutogenic diet.

3. Scorbutic guinea pigs fed a scorbutogenic diet supplemented by from 0.25–12.0 mg. ascorbic acid daily show rapid increases in serum alkaline phosphatase activity and rapid decreases when ascorbic acid is withdrawn.

4. Ascorbic acid does not activate the low serum phosphatase of scorbutic guinea pigs in vitro. The effect in vivo is due to an increase in enzyme production during recovery from scurvy.

5. There is no apparent change in serum "acid" phosphatase or in the serum inorganic phosphate of scorbutic guinea pigs.

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QUANTITATIVE DISTRIBUTION OF PHOSPHORUS AND CALCIUM IN CERTAIN FRUITS AND VEGETABLES

H. C. SHERMAN AND M. S. RAGAN
Department of Chemistry, Columbia University, New York

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The quantitative distribution of phosphorus and of calcium in the fruits and vegetables which we use as food is of scientific significance both with regard to our own nutrition and to our understanding of the nutritional processes of plants. In turn, any knowledge which we may gain of the quantitative behavior of these elements in plant nutrition should help in the planning and interpretation of studies of the extent to which the calcium and phosphorus contents of foods may be influenced by cultural conditions.

The idea — sometimes called Liebig's "law of the minimum" and apparently confirmed by Claude Bernard's dictum of the fixité of the internal environment — that the chemical composition is essentially constant for a given organ of a given species, is now seen to have been an over-simplification. Within a given species chemical composition may be measurably influenced both by environmental and genetic factors. Because of limitations of space we here cite but two of the recent investigators of such variations.

Miller ('38) while citing many observations to show that the older views were too rigid still states it as common knowledge that plants of different species grown under identical conditions differ in their elementary composition; and Hoagland and coworkers also show species differences in the relative amounts of chemical elements which plants absorb,

although the amount absorbed and its effect on growth and reproduction are also largely influenced by the relative concentrations, and the interactions, of the ions present (Hoagland, '40; Hoagland and Arnon, '41).

That natural and induced differences in chemical composition of tissues may be more significant than science had until recently supposed is a fact of both theoretical and practical importance. Also it is important that this fact be not exaggerated, as has happened when extreme instances were cited as if they were representative.

The investigation reported in the present paper deals (1) with presumably representative specimens of foods from various sources as they actually reach the consumer, i.e., with all inedible parts removed, and (2) with the quantitative distribution of the calcium and phosphorus of such foods among the structural parts of food plants.

Concentration of phosphorus in seeds

It is well known that plants tend to concentrate phosphorus in their seeds; calcium, in their leaves. Thus in presumably typical cases of the corn plant it has been found that at maturity 75% of the total phosphorus was in the seeds, while 58% of the total calcium was in the leaves (Latshaw and Miller, '24; Miller, '38).

Our studies here reported include (1) determinations of phosphorus in the seeds compared with the flesh (pulp and juice) of blackberries, raspberries, currants, grapes, tomatoes, okra, and squash; (2) studies of the calcium and phosphorus contents of the seeds versus the pods of four varieties of beans representing different stages of maturity; and (3) similar comparisons of the calcium and phosphorus contents of the flowerbuds (the first step toward formation of seeds) of broccoli in comparison with the adjacent leaves and edible portions of the stems of the same plants.

The data of the first of the three series of studies just mentioned are summarized in table 1.

All of the work summarized in table 1 — the gathering or purchase of the food, the preparation of the sample, and every step of the analytical determination — was done by the same person (M.S.R.) so that the "personal equation" was the same throughout. The specimens analyzed were presumably representative samples of the respective kinds of food as

TABLE 1

Phosphorus contents of entire edible portions of fruits, of the same with seeds removed, and of the seeds alone

DESIGNATION	PHOSPHORUS, PER CENT OF FRESH WEIGHT			PERCENTAGE OF TOTAL P IN SEEDS	WEIGHT OF SEEDS AS PER CENT OF WHOLE FRUIT
	Whole fruit	Without seeds	Seeds		
Blackberries I	0.034	0.018	0.066	64	33
" II	0.030	0.018	0.085	71	29
" III	0.027	0.013	0.086	75	30
" IV	0.028	0.015	0.082	61	21
" V	0.032	0.018	0.097	44	15
" VI	0.025	0.011	0.064	54	21
" VII	0.029	0.013	0.113	50	13
" VIII	0.031	0.015	0.083	72	27
" Average	0.030	0.013	0.085		
Raspberries I	0.036	0.027	0.070	25	13
" II	0.037	0.028	0.076	33	16
" III	0.035	0.028	0.076	30	14
" IV	0.032	0.025	0.066	31	15
" Average	0.035	0.027	0.072		
Currants	0.036	0.018	0.132	63	17
Grapes (Tokay)	0.021	0.020	0.065	7	2.4
Tomatoes I	0.029	0.024	0.203	14	2.1
" II	0.027	0.025	0.070	12	4.5
" III	0.020	0.017	0.074	16	4.4
" IV	0.027	0.024	0.094	14	4.1
" V	0.023	0.019	0.107	21	4.6
" VI	0.025	0.021	0.138	18	3.3
" VII	0.024	0.018	0.136	13	2.4
" Average	0.025	0.021	0.117		
Okra	0.060	0.053	0.112	25	14.0
Squash	0.021	0.014	0.038	56	32.0

actually reaching consumers under present-day marketing conditions. The foods in this series are all of kinds which may be eaten either with or without their seeds, so that the quantitative distribution of the phosphorus as between the flesh and seed is of scientific significance as bearing upon the amounts of phosphorus presumably available in human nutrition, and also the plant-nutrition problem of the extent of the concentration of the phosphorus of the fruit into its seed. In the cases of blackberries, raspberries, and tomatoes, where from four to eight entirely separate specimens of each of these three species of fruit were analyzed, the extent of the variations here found is presumably representative of the variability of these foods as they are actually encountered in the general food supply, and should give correspondingly more valid impressions of the reliability of the foods as sources of phosphorus in the nutrition of the human population than would the data of an investigation whose object was to find the greatest variation which could be discovered or experimentally induced.

Hence, even for the numbers of cases here reported, it is of interest to note the average deviations, and whether these are greater for the fruits with or without their seeds.

There follow brief statements regarding our findings upon different foods listed in the same order as in table 1.

Blackberries contained about two-thirds of their total phosphorus in their seeds, where the percentage of phosphorus was about five times as high as in the flesh (pulp and juice) of the same fruits. The average deviation was 0.0022 (7% of the mean) in the blackberries with, and 0.0029 (22% of the mean) in the berries without, their seeds. The phosphorus content without the seeds is therefore somewhat the more variable; but it doubtless represents more nearly the amount actually available in human nutrition.

In raspberries the per cent of phosphorus in the seeds was almost three times that in the pulp and juice, but less than one-third of the total amount of phosphorus in this fruit was in its seeds. The average deviation was about the same pro-

portion (only about 4%) of the mean value for the fruit with or without the seeds. Probably only a negligible part of the contents of the seed is nutritionally available to man.

In currants 63%, and in grapes only 7% of the total phosphorus was here found in the seeds.

Tomatoes, with their larger proportion of pulp and juice, contain therein well over four-fifths of their total phosphorus, and less than one-fifth in the seeds even though the concentration is six times as high in the seeds as in the flesh of the fruit. The average deviation was 9% of the mean phosphorus content of tomato with, and 11% of that of tomato without, the seeds. Tomatoes as they reach consumers, even in a large city drawing its supply from widely different sources, are thus of very satisfactorily constant phosphorus content.

In the single samples here analyzed, okra contained 25%, and summer squash 56%, of its total phosphorus in its seeds.

It is a safe generalization that foods should be analyzed without their indigestible seeds if the analysis is to show what the food actually furnishes to the nutritional process, and that the exclusion of the seeds widens the calcium/phosphorus ratio.

On the other hand, there are foods in whose development the seed becomes the part of most significance for our nutrition. Thus we eat snap beans when the seed is so immature that more than nine-tenths of the edible portion is pod and the calcium/phosphorus ratio of this entire edible portion is fairly wide (1.46 in the first item of table 2); whereas this ratio is narrow in the phosphorus-rich mature seed (0.18 in the last item of the same table).

Calcium and phosphorus in pods and seeds of beans

Calcium and phosphorus were determined in pods and seeds (ovules) of several varieties of beans, some of them at different stages of maturity. These data are summarized in table 2. As the differences are due in part to variety and in part to maturity, full discussion would require too much space. It suffices to note that the order of the specimens in the table is that of increasing maturity and that the last column clearly

TABLE 2

Calcium and phosphorus in pods and ovules of beans

DESCRIPTION	SEEDS, PER CENT OF TOTAL WEIGHT	WHOLE		PODS		SEEDS		PERCENTAGE OF THE TOTAL P IN THE SEEDS
		Ca %	P %	Ca %	P %	Ca %	P %	
Wax (early snap stage)	5½	0.057	0.039	0.063	0.030	0.053	0.147	22
Green stringless	7	0.044	0.037	0.045	0.024	0.046	0.139	32
" "	9½	0.043	0.039	0.046	0.032	—[1]	0.134	30
Cranberry, green	41	0.095	0.110	0.120	0.013	0.051	0.214	91
" yellow	44	0.112	0.113	0.167	0.012	0.055	0.203	93
" pink-striped	52	0.125	0.102	0.215	0.008	0.046	0.200	96
Black-eye	52	0.059	0.087	0.048	0.018	0.057	0.133	89
"	80	0.055	0.181	0.168	0.022	0.033	0.188	97

[1] Not determined.

shows a trend to increased concentration of phosphorus in the seed as it matures. The ratio of calcium to phosphorus is higher in the pods than in the seeds, with rather wide variations in a small proportion of the samples.

The apparent increase in calcium in the pods with maturity would indicate that the calcium migrated from plants to pods but not from pods to seeds. This association of high calcium content with tougher pods would seem to indicate increase of pectin and hemicellulose with maturity as found by other investigators (True, '22; Buston, '35).

Broccoli illustrates another type of case, three organs of differing calcium/phosphorus ratios being offered together in the edible portion; e.g., in the two specimens which were here analyzed for both calcium and phosphorus, the ratios were: Leaves , 3.4 and 3.7; edible stems, 1.7 and 3.2; flowerbuds, 0.5 and 0.4.

Distribution between leaves, flowerbuds, and edible stems of broccoli

Four samples of broccoli were purchased at different times in New York City. Including in the samples for analysis as much of the stem in each case as could reasonably be con-

sidered edible, and all the leaves belonging to this portion of the stem, the stems constituted nearly half, the leaves about one-fourth, and the flowers about one-fourth of the total material. In all cases the different parts were analyzed separately for phosphorus, and in two cases for calcium also. The results are summarized in table 3.

TABLE 3

Phosphorus (and calcium) in three edible parts of broccoli

SAMPLE NO. ELEMENT	ENTIRE EDIBLE PORTION	LEAVES	EDIBLE STEMS	FLOWER-BUDS	FLOWERBUDS AS PER CENT OF TOTAL E. P.	
					Weight	Phosphorus
I Phosphorus %	0.060	0.064	0.035	0.107	25	45
II Phosphorus %	0.063	0.070	0.040	0.104	24	40
Calcium %	0.110	0.237	0.068	0.052		
III Phosphorus %	0.056	0.055	0.033	0.102	23	43
Calcium %	0.118	0.202	0.105	0.043		
IV Phosphorus %	0.060	0.071	0.031	0.095	34	53

The mean phosphorus contents with their average deviations (and the latter as percentage of the former) are respectively: Whole edible portion, 0.060 ± 0.0018 (av.d. = 3.0%); leaves, 0.064 ± 0.0055 (av.d. = 8.5%); edible stems, 0.035 ± 0.0028 (av.d. = 8.0%); flowerbuds, 0.107 ± 0.0035 (av.d. = 3.4%). There is a well-marked concentration of phosphorus in the flowerbuds, and a still more pronounced concentration of calcium in the leaves.

Distribution of calcium in the edible portion of the orange [1]

Formerly, in compiling analyses from the literature, data for the edible portion and for the juice of oranges were tabulated together; but as data accumulated and especially when we were privileged to see unpublished findings of Thomas and Bailey on orange juice obtained by pressure alone, we were led to a study of the quantitative distribution of the calcium be-

[1] This part of the investigation was aided by a research grant to Columbia University by the Florida Citrus Commission.

tween the septa and the more fluid part (juice and soft pulp) of the edible portion. Our experimental findings on this point are summarized in table 4. The ten lots included oranges of large, medium, and small sizes and grown in different regions.

TABLE 4

Calcium in oranges: whole edible portion, juice, and septa

LOT NO.	CALCIUM CONTENT OF:			PERCENTAGE OF TOTAL CA IN SEPTA
	Whole E. P.	Juice with soft pulp	Septa	
	per cent	*per cent*	*per cent*	
I	0.044	0.011	0.164	70
II	0.046	0.012	0.183	75
III	0.044	0.011	0.180	77
→IV	0.040	0.012	0.159	75
V	0.046	0.011	0.118	72
VI	0.046	0.012	0.159	75
VII	0.045	0.011	0.156	75
VIII	0.034	0.015	0.081	70
IX	0.045	0.011	0.167	70
X	0.043	0.010	0.167	70
Average	0.043 ± 0.0027 [1]	0.012 ± 0.0010 [1]	0.153 ± 0.0218 [1]	73 ± 3 [1]

[1] Average deviation.

The average deviations of the calcium contents of the respective parts when stated as percentage of the mean in each case are only: in the whole edible portion, 6.3%; in the juice and pulp, 8.3%; in the septa, 14.2%. With such degrees of constancy in each part, the finding that the septa contain 73 ± 3% of the calcium of the whole edible portion of the orange may be regarded as definite. When one eats the entire edible portion of an orange (septa included) the calcium/phosphorus ratio, as illustrated by our determinations here reported for calcium and compiled data for phosphorus, is 2.0; whereas if the orange is taken as "juice" the ratio is about 0.8.

SUMMARY

Quantitative studies have been made of the relative amounts of phosphorus in the flesh and seeds of seven species of fruits;

in the pods and ovules of four varieties of beans; in the leaves, the edible stems, and the flowerbuds of broccoli; and of calcium in the same parts of broccoli, and in the flesh (juice and pulp) and septa of oranges.

In the course of this work, either phosphorus or calcium was determined in from four to ten entirely separate specimens each, obtained through ordinary marketing channels in a city supplied from widely varied sources, of twelve foods representing edible stems, leaves, flowerbuds, fruits, and edible seeds. In only one-sixth of these foods was the average deviation in phosphorus or calcium content as much as one-tenth of the mean.

Allowing for the extent of the variations encountered in fruits and vegetables as actually produced and marketed, the following findings may be regarded as sufficiently established:

In such "small fruits" as blackberries, currants, and raspberries, the phosphorus content is significantly different when the "edible portion" of the fruit is analyzed with and without the seeds.

In the grapes here studied, the seeds show three times as high a percentage of phosphorus as the flesh, but constitute so small a proportion of the whole fruit as scarcely to influence its average composition.

In tomatoes, okra, and squash (fruits commercially classified as vegetables) the phosphorus content of the edible portion was measurably influenced by the inclusion or exclusion of the seeds. To what extent human beings can assimilate the phosphorus of these seeds remains to be studied.

The quantitative aspect of the transfer of phosphorus from pods to ovules in maturing beans is found to be striking.

Also well marked is the concentration of calcium in the septa of oranges as compared with the juice. The latter, even when carrying the softer portions of the pulp, was found to contain only 27% of the calcium of the edible portion.

The data here reported will also add to the material available to investigators of the natural variability of foods.

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EFFECT ON GROWTH AND CALCIUM ASSIMILATION OF CITRIC ACID — POTASSIUM CITRATE MIXTURES [1]

CAROLINE SHERMAN LANFORD
Department of Chemistry, Columbia University, New York City

(Received for publication September 11, 1941)

In an earlier paper (Lanford, '39), experiments were reported in which the addition of a moderate amount of orange juice to a wheat-and-milk diet caused the assimilation by rats of a larger proportion of the calcium in the food mixture. The present series was planned to test whether the mixture of citric acid and citrates in the orange juice was responsible for its effect in increasing the percentage retention of dietary calcium. It seems clear that this was not the case, under the conditions of our experiment. However, inasmuch as other investigators have since reported beneficial effects of citric acid-citrate mixtures on rickets, both experimental (Shohl, '37; Hamilton and Dewar, '37) and clinical (Shohl and Butler, '39; Siwe, '38), and in some (Day, '40; Hathaway and Meyer, '39), but not all (Day, '40), circumstances, on bone ash, it seems desirable to here report our findings.

EXPERIMENTAL

The basal diet was the same as in the experiment with orange juice. It consisted of 5 parts of ground whole wheat and 1 part of dried whole milk, with added sodium chloride equivalent to 2% of the wheat; a mixture which was found to contain 0.202% of calcium, and may be inferred from earlier studies to supply about 0.44% of phosphorus and to have an energy value of 3.73 kilocalories per gram. (The basal diet itself contained citrate equivalent to about 0.02% as citric acid.)

[1] This research was aided by a grant from the Florida Citrus Commission.

The citric acid-potassium citrate supplement was given as 5 cc. daily [2] of a solution [3] containing 1.6% total (free plus combined) citric acid, of which, in accordance with the findings of Hartmann and Hillig ('34) for orange juice, about three-fourths was free (titratable) acid. The amount of citric acid and citrates thus fed to the experimental animals was twice that provided by the orange juice of the earlier series.

Late each afternoon, all animals were given slightly less of the basal diet than they would normally consume overnight, mixed, in the case of the controls, with 5 cc. of distilled water, and, in the case of the experimental animals, with 5 cc. of the acid-citrate solution.[4] This portion was almost invariably found to have been completely eaten by the next morning, when the dry mixture was again offered ad libitum. In the few exceptional instances, the portion remaining had to be scraped loose from the walls of the container, after which it was promptly consumed by the animal. By far the greater part of the basal diet was taken in the moistened form.

The pairs of animals on which this comparison is based consisted of littermates of the same sex, matched as to initial body weight and build. At the start of the experiment the difference in body weight between the two members of each pair averaged 0.5 gm. for the nine pairs of males and 1.0 gm. for the ten pairs of females. All animals were selected from families which had been maintained through several generations on the basal diet. As in the earlier series, the rats were 28 days of age when the experiment was begun, and at 60 days of age were killed and analyzed as described elsewhere (Lanford, '39).

[2] Except on Sundays and on the day preceding that on which the animals were to be killed.

[3] Prepared by dissolving 1.31 gm. of crystalline citric acid ($C_6H_8O_7 \cdot H_2O$) and 0.48 gm. of crystalline potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) in 100 cc. of solution.

[4] In this series, no attempt was made to dilute the basal diet of the controls with a neutral substance supplying the same number of calories as did the citrate solution, since the latter amounted to less than 0.7% of the total caloric intake, falling therefore well within the experimental error in measuring food consumption.

RESULTS AND DISCUSSION

Interpretation of data. Besides the directly measurable values for food intake, initial body weight, gain in weight during the experimental period, and total body calcium at 60 days of age, given for each animal in table 1, computations based on these data were made for (a) gain per 1000 kilocalories consumed, (b) total calcium retained during the experimental period, and (c) calcium retained as percentage of the intake. In computing (a), an energy value of 471 kilocalories per mol was taken for citric acid (from the International Critical Tables). To determine calcium retention, the calcium content of each animal at the start of the experimental period was estimated from analyses of other 28-day-old animals of the same hereditary and nutritional background, the male and female animals showing respectively an average of 0.714% and 0.738% of calcium in net weight, the net weight averaging 5.0 gm. less than the live weight for both sexes at this age.

The significance of the data has been assessed statistically in two ways: comparison being made both between the corresponding sex and dietary groups, each considered as a unit (with the results in table 1), and between the individual members of each pair, as in table 2. Only by the latter procedure is the advantage of careful initial matching of the pairs fully appreciable in more clear-cut results. Thus, for example, when the data on weight gain from 28 to 60 days of age are treated by the former procedure the difference in performance between the animals which did and those which did not receive the citrate mixture appears nonsignificant; whereas, by the latter treatment an unmistakable difference is shown.

Effect of citric acid — potassium citrate mixtures on calcium retention. Under the conditions of this experiment, administration of a mixture of citric acid and potassium citrate in the molar proportions of 3:1 (thus simulating the citrate buffer mixture of orange juice) was without measurable effect on the percentage retention of the calcium of a wheat-and-milk diet.

TABLE 1

Record of individual rats, with and without acid-citrate supplement.

RAT NO. AND DIET GROUP		FOOD INTAKE		BODY WEIGHT			BODY CALCIUM		
		Basal diet	Citric acid-citrate solution	At 28 days	Gain, 28–60 days		At 60 days	Increase, 28–60 days	
		gm.	*cc.*	*gm.*	*gm.*	*gm./1000 kg.cal.*	*gm.*	*gm.*	*% of intake*
Males									
80689	acid-citrate	258.5	130	40.7	81.3	83.87	0.7449	0.4900	93.83
80690	control	230.0		40.2	64.8	75.53	0.6895	0.4382	94.32
80692	acid-citrate	242.0	130	37.4	75.6	83.28	0.6989	0.4761	97.40
80691	control	253.5		39.0	81.0	85.66	0.7464	0.5036	98.34
80813	acid-citrate	246.0	135	38.6	71.4	77.37	0.7029	0.4632	93.22
80812	control	243.5		38.6	71.4	78.61	0.6935	0.4538	92.25
80829	acid-citrate	284.0	130	43.6	83.4	78.36	0.7855	0.5101	88.92
80830	control	224.5		43.1	60.9	72.73	0.6828	0.4110	90.63
80930	acid-citrate	286.0	135	42.0	87.0	81.15	0.7722	0.5080	87.93
80931	control	285.5		41.5	92.5	86.86	0.7650	0.5044	87.46
80950	acid-citrate	291.0	130	39.4	86.6	79.41	0.7589	0.5133	87.33
80952	control	274.5		38.7	78.3	76.47	0.7130	0.4734	85.37
80960	acid-citrate	273.0	130	40.3	77.7	75.92	0.7248	0.4728	85.73
80961	control	228.5		39.9	60.1	70.52	0.6465	0.3973	86.07
80963	acid-citrate	251.0	130	35.1	75.9	80.63	0.6721	0.4572	90.18
80962	control	219.0		35.5	63.5	77.73	0.6048	0.3870	87.48
80979	acid-citrate	242.5	130	39.5	70.5	77.51	0.7105	0.4642	94.77
80978	control	200.0		39.5	52.5	70.38	0.6235	0.3772	93.37
Mean	*acid-citrate*	*263.8*		*39.6*	*78.8*	*79.72*	*0.7301*	*0.4839*	*91.03*
					±1.4	*±0.60*		*±0.0049*	*±0.87*
	control	*239.9*		*39.6*	*69.4*	*77.17*	*0.6850*	*0.4384*	*90.59*
					±2.8	*±1.32*		*±0.0111*	*±1.00*
Females									
80693	acid-citrate	238.5	130	39.1	62.9	70.30	0.7018	0.4502	93.44
80695	control	227.5		38.4	62.6	73.77	0.6743	0.4276	93.04
80696	acid-citrate	235.0	130	36.1	62.9	71.34	0.6822	0.4525	95.32
80694	control	236.5		37.9	60.1	68.13	0.6917	0.4487	93.93
80699	acid-citrate	253.0	130	43.5	65.5	69.04	0.7468	0.4625	90.49
80700	control	219.5		42.0	51.0	62.29	0.6740	0.4008	90.39
80703	acid-citrate	199.5	130	36.8	48.2	64.34	0.6112	0.3765	93.42
80702	control	206.5		39.5	45.5	59.08	0.6554	0.4008	96.09
80815	acid-citrate	208.0	135	41.0	49.0	62.73	0.6473	0.3814	90.77
80816	control	227.5		40.0	56.0	65.99	0.6812	0.4227	91.97
80818	acid-citrate	234.5	135	38.9	64.1	72.84	0.6893	0.4389	92.66
80817	control	229.0		39.0	60.0	70.24	0.6828	0.4317	93.32
80935	acid-citrate	244.5	135	39.6	60.4	65.85	0.6882	0.4329	87.65
80934	control	212.0		39.5	48.5	61.33	0.6218	0.3672	85.75
80954	acid-citrate	244.0	130	39.0	65.0	71.02	0.6778	0.4269	86.61
80953	control	249.5		38.0	66.0	70.92	0.6746	0.4311	85.54
80955	acid-citrate	251.5	130	37.2	69.8	74.01	0.6936	0.4560	89.76
80957	control	233.5		37.7	60.3	69.23	0.6505	0.4092	86.75
80956	acid-citrate	251.0	130	36.9	67.1	71.28	0.6656	0.4302	84.85
80958	control	235.0		36.0	64.0	72.10	0.6512	0.4224	87.85
Mean	*acid-citrate*	*236.0*		*38.8*	*61.5*	*69.28*	*0.6804*	*0.4308*	*90.50*
					±1.5	*±0.82*		*±0.0063*	*±0.71*
	control	*227.6*		*38.8*	*57.4*	*67.31*	*0.6658*	*0.4162*	*90.46*
					±1.5	*±1.05*		*±0.0048*	*±0.80*

Since the citrates were fed in this experiment at twice the level provided by the orange juice supplement of the earlier study, which unmistakably increased the percentage utilization of calcium on the same basal diet, it seems clear that the beneficial effect of the orange juice cannot be explained in terms of the citrates which it contains. It seems highly improbable that too great amounts of citric acid and citrates were provided by the supplement in this experiment (twice as large as in the orange juice study), and that an increase in the percentage retention of calcium might have been observed at lower levels of intake. The investigators already mentioned who found a beneficial effect of citrates on bone development and other aspects of calcium metabolism under

TABLE 2

Differences between animals receiving citric acid — citrate mixture and own littermate controls.

	DIFFERENCE, CITRATE ANIMAL MINUS CONTROL	
	Mean	Probable error of mean
Gain in body weight, 28–60 days, gm.	+6.6	±1.34
Gain per 1000 kilocalories, gm.	+2.30	±0.635
Calcium retained, total, gm.	+0.0292	±0.0065
Calcium retained, per cent of intake	+0.23	±0.21

more adverse conditions gave many times more citrate than were here used. It is true that, under less favorable conditions for calcium assimilation, the percentage retained might have been improved by the citrate supplement. However, for our observation that, with the same basal diet supplying the calcium, orange juice increased the fraction of that element which was retained whereas the citric acid-citrate supplement did not, the only interpretation seems to be that citrus juice possesses some additional property which favors calcium assimilation.

This effect of the orange juice appears not to have been due to its preponderance of base-forming elements (equivalent to 0.23 cc. N base in the amount given daily), since the citrate

supplement of the present study had a greater "potential alkalinity" (equivalent of 0.31 cc. N base per day), yet was without effect on the proportion of dietary calcium retained.

The total retention of calcium during the experimental period was significantly higher in the animals receiving citrate, who also ate more of the basal diet and made greater gains in weight than did the control group.

Effect of acid-citrate on growth. The gain in weight of the males receiving the citrate supplement averaged 14%, and that of the females, 7% above those of the control groups (all of the animals having been allowed food essentially ad libitum, as explained above). When matched littermates are compared, the difference in growth in favor of the individuals given citrate appears significant, averaging 6.6 gm. with a probable error of 1.34 gm.

Correspondingly, the gain per 1000 kilocalories averaged 2.30 ± 0.635 gm. higher in the animals which received the supplementary citrate. Since, however, a strongly positive correlation between actual gain in weight and gain per 1000 kilocalories was observed in both this (see table 3) and the earlier study (Lanford, '39) a greater gain per 1000 kilocalories in the citrate group should have been expected ipso facto from the greater total gain.

Interrelationships. In table 3 are given values for Pearson's coefficient, r, of correlation between several pairs of variables

TABLE 3

Coefficients of correlation, r, for various possible interrelationships, with probable error of r.

GROUP	MALES, CONTROL	MALES, CITRATE	FEMALES, CONTROL	FEMALES, CITRATE
	$r \pm p.e.$	$r \pm p.e.$	$r \pm p.e.$	$r \pm p.e.$
Ca retained, per cent of intake	—0.06	—0.21	—0.26	—0.31
vs. gain, 28–60 days	±0.222	±0.212	±0.196	±0.190
Ca retained, per cent of intake	+0.07	—0.32	—0.01	—0.09
vs. body weight at 28 days	±0.221	±0.199	±0.210	±0.208
Gain per 1000 kilocalories	+0.80	+0.26	+0.89	+0.82
vs. gain, 28–60 days	±0.080	±0.207	±0.043	±0.069
Body Ca, per cent of net weight	—0.80	—0.75	—0.81	—0.72
vs. gain, 28–60 days	±0.080	±0.097	±0.072	±0.101

whose possible interrelationships are of interest to this discussion. Admittedly, nine or ten comparable cases comprise a group too small for a fully satisfactory test of correlation, especially if the degree of correlation is low. Consequently, all that may be concluded from these figures regarding percentage retention of calcium as possibly affected by body weight at 28 days or by rate of gain in weight is that, if there be any interrelationship, it is probably of low order and doubtful significance. On the other hand, three of the four groups afford striking illustration of the tendency noted elsewhere (Lanford, '39) for a rapid gain in weight to be associated in the same individual with a relatively large gain per 1000 kilocalories. Likewise, the high degree of negative correlation found here between gain in weight and percentage of calcium in the body confirms the same finding in the orange juice experiment, and related observations (Lanford, Campbell and Sherman, '41) on animals during the period of rapid growth.

SUMMARY

Administration of a mixture of citric acid and potassium citrates, in approximately the proportions and quantities which would be supplied by 10 cc. of fresh orange juice daily, increased the rate of growth and the total calcium retention of young rats on a wheat-and-milk diet, but, unlike the orange juice tested earlier in a similar way, had no appreciable effect on the percentage of the dietary calcium which was retained in the body of the growing animal. It thus appears that orange juice favors calcium assimilation in growth through some property in addition to its content of citric acid and citrates and its preponderance of base-forming elements.

The assistance of Grace Guldin Stroup in these studies is gratefully acknowledged.

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THE STATE OF VITAMIN A IN THE LIVER OF THE RAT

II. THE EFFECT OF FEEDING THE VITAMIN OVER EXTENDED PERIODS [1]

E. LEB. GRAY AND JOHN D. CAWLEY

The Research Laboratories of Distillation Products, Inc., Rochester, New York [2]

THREE FIGURES

(Received for publication September 30, 1941)

Previous work (Gray, Hickman, and Brown, '40) has shown that no matter in what form vitamin A may be fed to the rat, it appears in the liver esterified in a more or less specific manner. However, the breadth of the elimination curve of this vitamin A present in liver shows that several esters must be present. In that work, high doses of the vitamin were fed over short periods of time. As that experiment represented an extreme case, it seemed desirable to investigate the liver when the rats had been fed more nearly normal doses over extended periods.

Four groups of rats of the Sprague-Dawley strain, containing four to six animals per group, were therefore placed at weaning on the Sherman B diet (Sherman and Campbell, '24) which was supplemented with 500 units of vitamin A daily.[3] The four groups were fed dogfish liver oil, dogfish liver oil

[1] This paper was read before the Biological Section of the American Chemical Society at the 101st meeting, April 9, 1941.

[2] Communication No. 23 from the Laboratories of Distillation Products, Inc.

[3] Elizabeth F. Brown of the Department of Zoology, University of Rochester, supervised the care and feeding of the rats. This work was part of an experiment to determine the toxicity of vitamin A, the results of which are to be published elsewhere. The Sherman B diet is composed of 66% whole wheat, 33% whole milk powder, and 1% sodium chloride.

concentrate, shark liver oil, and shark liver oil concentrate, the concentrates being prepared by distillation of the respective oils. The potencies of the oils in U.S.P. units were: Dogfish liver oil, 16,000; dogfish liver oil concentrate, 236,000; shark liver oil, 105,000; shark liver oil concentrate, 619,000.

Each oil was diluted with cottonseed oil [4] so that a drop contained 100 U.S.P. units of vitamin A. Dosing was started at weaning with 1 drop per day, and the dose gradually increased to 5 drops daily. The feeding period extended through 112 days, each rat receiving a total of 52,000 units.

At the conclusion of the feeding period the animals were sacrificed and the livers removed and immediately placed at —50° C., at which temperature they were kept until used. For examination the livers were thawed, ground with anhydrous sodium sulphate and extracted in a Soxhlet apparatus first with 95% alcohol, then with ether. The solvents were removed under nitrogen and potencies and spectra of the extracts determined. The potencies by the antimony trichloride reaction were determined on an Evelyn photoelectric colorimeter calibrated in U.S.P. units with a vitamin A ester concentrate of known extinction coefficient, using the conversion factor 2110 to convert $E^{1\%}_{1\ cm.}$ (328 mu) to U.S.P. units. Ultraviolet absorption spectra were determined in cyclohexane solution on a Hilger medium quartz spectograph equipped with a Spekker photometer. Potencies as determined colorimetrically and from the extinction coefficient at 328 mu, using the conversion factor 2110, showed a maximum deviation of 9%. Table 1 gives a summary of these determinations. The recovery of the vitamin is in line with that obtained in the previous work. Distilled shark liver oil concentrate representing a recovery of 72% is, however, definitely higher than the others, but since it represents a single experiment, too much weight cannot be attached to this figure.

The extracts were taken up in ether and washed with aqueous alkali and water. At this point emulsions extremely difficult to deal with were encountered and some mechanical

[4] Wesson.

TABLE 1

The recovery of vitamin A from the liver of the rat after feeding the vitamin over an extended period.

GROUP	NO. OF RATS	SUBSTANCE FED	WEIGHT OF LIVERS		WEIGHT OF EXTRACT	POTENCY OF EXTRACT	TOTAL VITAMIN A FED	TOTAL VITAMIN A REC.	RECOVERY
			Total	Av. Wt. per rat					
			gm.	*gm.*	*gm.*	*I.U. per gm.*	*I.U.*	*I.U.*	%
VII	6	Dogfish liver oil	52.5	8.7	3.40	39,000	312,000	133,000	43
IX	4	Dogfish liver oil conc.	38.5	9.6	2.56	34,900	208,000	89,000	43
VI	6	Shark liver oil	52.5	8.7	2.94	60,700	312,000	178,000	57
IV	5	Shark liver oil conc.	46.5	9.3	2.72	68,500	260,000	186,000	72

loss of vitamin occurred. Unfortunately, an accident occurred here resulting in the complete loss of the extract from the dogfish liver oil concentrate, so that no distillation data are available on this group. In the remaining groups the ether solution was dried over anhydrous sodium sulphate, the ether removed, and the residue dissolved in 25 gm. of constant yield oil and 25 gm. of corn oil residue[5] and subjected to analytical

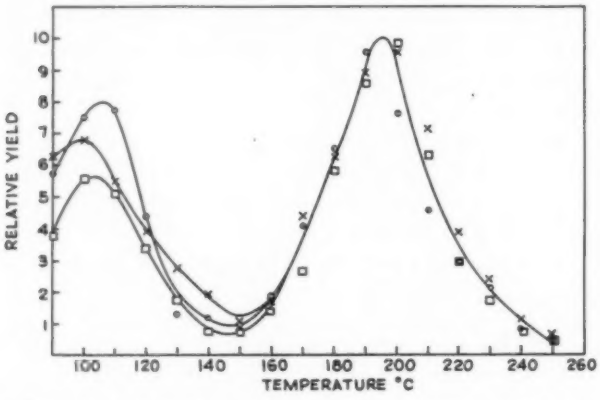

Fig. 1 Vitamin A elimination curves of liver extracts after feeding the vitamin over an extended period:
○ from rats fed dogfish liver oil
× from rats fed shark liver oil
□ from rats fed shark liver oil concentrate

molecular distillation (Hickman, '37). The results of the distillations are plotted in figure 1. The three curves are plotted together, since examination of the individual curves showed that all three were identical within the limits of error of the method. Inspection of figure 1 shows a major maximum at 195° C. with a somewhat smaller maximum at 105° C. Ultraviolet absorption curves were determined on the fractions distilling at each maximum and a representative curve for each is shown in figures 2 and 3. Figure 2 is the absorption curve

[5] A high-boiling fraction, free from vitamin A, obtained from the complete distillation of corn oil.

of the material distilling at 195° C., and is the type of curve obtained for a good quality fish liver oil or concentrate. The absorption curve obtained from fractions distilling at the 105° C. maximum, shown in figure 3, is that of cyclized vitamin A (Castle, Gillam, Heilbron, and Thompson, '34; Embree, '39).

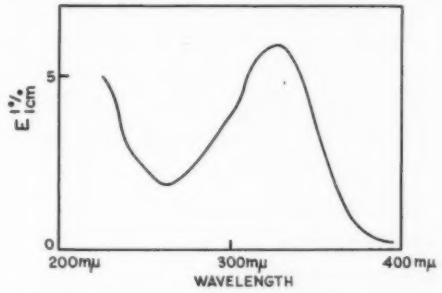

Fig. 2 Typical absorption spectrum of the fraction distilling at 195° C.

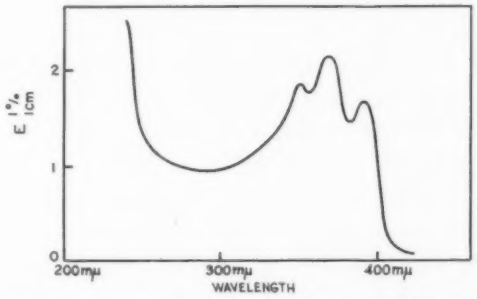

Fig. 3 Typical absorption spectrum of the fraction distilling at 105° C.

DISCUSSION

The complexity of the ester fraction obtained in the short-term feeding experiment is to be noted in comparison with the present experiments. Here the ester fraction is obviously that of a single substance, possibly the palmitate. This transition of vitamin A in the liver from the state of esterification with many fatty acids to that of one fatty acid takes place

gradually. When the vitamin is fed for a period of 60 days, the curve is intermediate in position, showing the presence of more than one ester, but not nearly so complex as when the animal is suddenly flooded with the vitamin (Morgareidge, Gray, and Cawley, unpublished work). Thus there is a selective process going on in the animal which finally results in the esterification of the vitamin with a single fatty acid. Furthermore, this process is extremely slow, taking well over 60 days for completion when the vitamin is fed at a level of 500 units daily. Thus the rat has a definite limit as regards his normal capacity for taking care of vitamin A. Experiments to determine toxicity, and employing much higher levels of the vitamin, bear this out.

Since the completion of this work, one of us (J.D.C.) has found that the cyclized vitamin A present in the extracts is an artifact, arising from the use of alcohol in the extraction of the livers. Details of this phenomenon will be reported more fully elsewhere, but it may be stated here that the cyclization occurs only with the vitamin esters, not the free alcohol, and is not due to the presence of anhydrous sodium sulphate or tissue; cyclization takes place if a vitamin A ester, either synthetic or naturally occurring, is merely refluxed with an alcohol. Since all of several pure synthetic esters have shown this reaction to the same degree, the only effect has been to diminish the amount of ester present, without distorting the ester picture.

CONCLUSIONS

1. When vitamin A is fed in moderate amounts over long periods of time, the vitamin A stored in the liver is gradually transformed from its combination with several fatty acids to a union with but one.

2. Cyclized vitamin A is formed from vitamin A esters on refluxing with alcohols.

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THE RATE OF INCREASE OF BLOOD PLASMA ASCORBIC ACID AFTER INGESTION OF ASCORBIC ACID (VITAMIN C)[1]

E. NEIGE TODHUNTER,[2] RUTH C. ROBBINS AND JENNIE A. McINTOSH
Division of Home Economics, Agricultural Experiment Station, State College of Washington, Pullman

ONE FIGURE

(Received for publication October 25, 1941)

In mass studies of vitamin C nutrition it is not always practical to obtain fasting blood samples. This study was undertaken primarily to ascertain how rapidly and to what extent ascorbic acid ingested with breakfast would cause a rise in blood plasma ascorbic acid above the fasting level; and also to determine the effect of different food sources of the vitamin, and other possible factors, in raising the plasma level.

EXPERIMENTAL

Subjects

The subjects were five college women of the following heights and weights: A, 167.6 cm., 60.78 kilo.; B, 160.0 cm., 45.81 kilo.; C, 174.0 cm., 58.97 kilo.; D, 175.9 cm., 71.67 kilo.; E, 163.8 cm., 58.97 kilo.

[1] Published as scientific paper no. 502, College of Agriculture and Agricultural Experiment Station, State College of Washington, Pullman.

Presented in part at the annual meeting, American Institute of Nutrition, Chicago, April, 1941.

This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

[2] Present address: Department of Nutrition, School of Home Economics, University of Alabama, Tuscaloosa, Alabama.

Plan of experiment

The ascorbic acid content of blood plasma was determined on samples obtained just before breakfast which was eaten at 8:00 a.m. Breakfast consisted of buttered whole-wheat toast and coffee, and 50 mg. of ascorbic acid which was taken either in the crystalline form [3] or in an equivalent amount of either strawberries, orange juice, orange sections, or raw cauliflower. Blood samples were then taken at 8:15, 8:30, and each succeeding half-hour up to and including noon. For each subject, determinations were made on three different mornings with each supplement.

Ascorbic acid determinations

From 0.6 to 0.8 ml. of blood was obtained from the finger tip, and duplicate or in some cases triplicate analyses of the plasma were made. Analyses were made by the micro-method of Farmer and Abt ('36), (tables 1 and 2) and in the later part of the study some analyses were by the Evelyn photoelectric colorimetric micro-method of Mindlin and Butler ('38) with the modification suggested by Bessey ('38) for correction for turbidity.

Comparisons were made of ascorbic acid content of plasma of capillary blood obtained at the same time from the ear and from the finger-tip of the same subject and they were found to give comparable values as shown by the following data. The values are in milligrams of ascorbic acid per 100 ml. plasma:

SUBJECTS:	A	B	C	D	E	F
Ear	1.01	1.18	1.09	1.09	0.92, 0.88	0.76
Finger tip	1.05	1.18	1.07	1.11	0.92, 0.86	0.74

In our laboratory the maximum error from possible difficulty in determining the end-point by the micromethod of Farmer and Abt ('36) has been found to be 0.08 mg. per 100 ml. blood plasma so that differences of less than 0.08 mg. are not considered as indicative of change in the plasma value.

[3] We are indebted to Merck and Company, Rahway, New Jersey, for a generous supply of pure ascorbic acid.

Each blood sample was analyzed without delay but studies were also made to determine the extent of possible changes in ascorbic acid content if samples were allowed to stand in the refrigerator at 11° C. for varying lengths of time at any stage in the procedure. Whole blood with an original value of 1.41 mg. ascorbic acid and analyzed after 3¾ hours was found to contain 1.35 mg. per 100 ml. of plasma; this difference is not significant. Other samples of whole blood with initial values of 1.23 and 1.25 mg. after 2 hours' storage showed values of 1.14 and 1.20 mg. respectively. Samples of whole blood which were centrifuged and stored with the plasma remaining in contact with the corpuscles showed no change in ascorbic acid content when analyzed after 2, 3, and 4 hours but one sample with an initial value of 0.92 mg. after 6½ hours' storage showed a value of 0.71 mg., a loss of 23%. Similarly, some ten different samples of deproteinized plasma stored in the refrigerator at 11° C. for from 2 to 5 hours showed no appreciable change in ascorbic acid content.

The ascorbic acid contents of the fruits and the cauliflower were determined by the micromethod using the Evelyn photoelectric colorimeter (Bessey, '38). The average ascorbic acid values obtained were: orange juice 0.49 mg. per milliliter (fourteen samples); orange flesh 0.49 mg. per gram (ten samples); strawberries 0.64 mg. per gram (eighteen samples); and cauliflower 0.50 mg. per gram (five samples; only the florets of the cauliflower were used).

RESULTS

Influence of ingested ascorbic acid on blood plasma level

When ascorbic acid was taken in the crystalline form at 8:00 a.m. the blood plasma level showed a slight increase by 8:30 a.m. in some cases, and a definite increase by 9:00 a.m. for all subjects, attaining a maximum by 9:30 and returning to the original fasting level by 11:00 or 11:30 a.m. The same subjects consistently showed the same general picture of absorption rate on each of the three different mornings that

TABLE 1

Ascorbic acid content of blood plasma after ingestion of 50 mg. ascorbic acid or an equivalent amount from orange juice and strawberries.

SUBJECT	SUPPLEMENT	MG. ASCORBIC ACID PER 100 ML. PLASMA AT GIVEN HOUR IN A.M.										MAXIMUM INCREASE IN PLASMA VALUE
		7:59	8:15	8:30	9:00	9:30	10:00	10:30	11:00	11:30	12:00	
												mg.
	crystalline ascorbic acid	1.38	1.41	1.41	1.56	1.63	1.58	1.50	1.46	1.43	1.41	0.25
		1.44	1.44	1.44	1.52	1.62	1.60	1.52	1.50	1.44	1.44	0.18
		1.29	1.31	1.29	1.43	1.61	1.52	1.38	1.29	1.29	1.26	0.32
A	orange juice	1.20	1.20	1.20	1.32	1.36	1.46	1.36	1.28	1.20	1.20	0.26
		1.34	1.34	1.34	1.43	1.64	1.60	1.51	1.43	1.43	1.34	0.30
		1.02	0.97	0.97	1.14	1.32	1.14	1.06	1.06	0.97	0.97	0.30
	strawberries	1.22	1.26	1.26	1.26	1.38	1.51	1.38	1.30	1.26	1.26	0.29
		1.10	1.10	1.10	1.10	1.20	1.34	1.24	1.20	1.10	1.10	0.24
		1.06	1.06	1.06	1.06	1.26	1.40	1.26	1.18	1.10	1.06	0.36
	crystalline ascorbic acid	1.06	1.04	1.12	1.32	1.36	1.32	1.28	1.14	1.16	1.12	0.30
		1.46	1.41	1.45	1.67	1.70	1.58	1.50	1.41	1.43	1.41	0.24
		1.10	1.10	1.20	1.20	1.38	1.31	1.24	1.10	1.08	1.10	0.28
B	orange juice	1.14	1.14	1.23	1.32	1.41	1.32	1.28	1.23	1.18	1.14	0.27
		1.09	1.09	1.09	1.26	1.34	1.22	1.18	1.14	1.09	1.09	0.25
		1.09	1.09	1.09	1.22	1.26	1.30	1.18	1.14	1.09	1.09	0.21
	strawberries	1.34	1.34	1.34	1.34	1.47	1.64	1.51	1.43	1.43	1.38	0.30
		1.06	1.10	1.06	1.06	1.18	1.32	1.23	1.18	1.14	1.10	0.26
		1.23	1.19	1.23	1.32	1.41	1.50	1.41	1.32	1.32	1.23	0.27
	crystalline ascorbic acid	1.21	1.23	1.23	1.32	1.50	1.41	1.34	1.26	1.23	1.23	0.29
		0.84	0.84	0.92	1.01	1.09	1.01	0.96	0.92	0.84	0.84	0.25
C	orange juice	1.00	1.04	1.04	1.28	1.20	1.12	1.10	0.96	0.96	0.96	0.28
		1.09	1.18	1.18	1.26	1.38	1.34	1.26	1.18	1.09	1.01	0.29
		0.97	0.97	1.01	1.06	1.23	1.18	1.06	1.06	1.02	0.97	0.26
	strawberries	1.43	1.43	1.38	1.43	1.51	1.68	1.60	1.51	1.51	1.43	0.25
		1.06	1.06	1.06	1.06	1.18	1.32	1.28	1.14	1.14	1.06	0.26
		1.06	1.06	1.06	1.14	1.23	1.32	1.18	1.14	1.14	1.10	0.26
	crystalline ascorbic acid	0.92	0.88	0.92	1.10	1.10	1.20	1.04	0.99	0.94	0.90	0.28
		0.94	0.92	0.94	1.04	1.10	1.04	1.02	0.97	0.94	0.97	0.16
		0.88	0.88	0.97	1.08	1.06	1.06	0.97	0.88	0.88	0.90	0.20
D	orange juice	1.06	1.06	1.14	1.23	1.23	1.14	1.14	1.06	1.06	1.06	0.17
		1.06	1.10	1.06	1.18	1.32	1.23	1.23	1.14	1.06	1.06	0.26
		1.08	1.06	1.06	1.23	1.32	1.21	1.14	1.14	1.06	1.06	0.24
	strawberries	1.06	1.06	1.06	1.10	1.23	1.36	1.32	1.23	1.14	1.14	0.30
		1.09	1.09	1.09	1.18	1.26	1.30	1.26	1.18	1.14	1.09	0.21
		1.18	1.18	1.18	1.18	1.26	1.38	1.34	1.26	1.18	1.18	0.20

determinations were made; and for four of the subjects of differing height and weight there was close agreement in the rate and time of reaching the maximum of absorption of the ascorbic acid. These data are summarized in table 1. The extent of the increase in plasma value was not always the same for each subject but in all cases the same general trend was observed with increases ranging from 0.16 to 0.32 mg. per 100 ml. blood plasma.

Some foods as a source of ascorbic acid

When either orange juice or sections of whole orange was taken in amounts to supply 50 mg. ascorbic acid, the maximum increases in plasma ascorbic acid levels were comparable to those obtained when crystalline ascorbic acid was ingested and the time of reaching the peak of increase was also similar (table 1 and fig. 1). When strawberries were the source of ascorbic acid the peak of the curve was reached in 2 hours and when raw cauliflower was taken, the time of maximum increase in plasma values was delayed until 10:30 a.m. with return to the original fasting level by 11:30 a.m. (fig. 1). Those fruits or vegetable which have considerable cellular material and therefore are more slowly digested were thus found to give a delayed response in blood plasma increase.

Influence of iron salts

When iron salts, in the form of 6 grains of ferrous sulphate,[4] or an equivalent amount of iron as ferric ammonium citrate were taken at the same time as the 50 mg. dose of ascorbic acid, the increase in plasma ascorbic acid was apparently uninfluenced for two subjects and was somewhat delayed for one subject. A typical curve for one subject is shown in figure 1.

Farmer, Abt and Aron ('40) reported that 6 grains or more of ferrous sulphate caused a sharp drop in plasma ascorbic acid level; however the iron salts were given over a period of 20 days and doses smaller than 6 grains daily were found to be ineffective.

[4] Feosol.

Influence of high fat intake

In order to determine whether a high intake of fat such as might be taken at breakfast would affect the plasma level, 50 gm. of butter were eaten with the standard breakfast at 8:00 a.m. This amount was calculated to be a possible intake if eggs, bacon, cream and butter were eaten at breakfast. A typical curve for one subject is shown in figure 1; subjects A

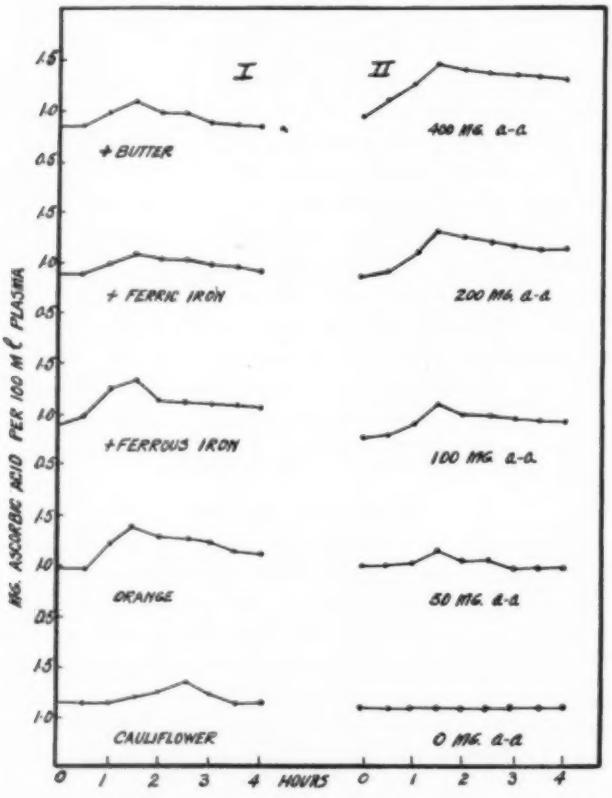

Fig. 1 Typical curves showing plasma content of ascorbic acid; I, after ingestion of 50 mg. of the vitamin as cauliflower or orange, or in the crystalline form plus butter, or plus ferrous or ferric iron; II, one subject with varying intakes of crystalline ascorbic acid.

and B showed the same general trend as previously noted with the peak of the curve being reached at 9:30 a.m., and for subject D the peak was attained at 10:30 a.m.

Influence of fasting level of plasma ascorbic acid on the extent of increase in response to a 50 mg. intake

The original fasting level may be expected to influence the extent to which the plasma ascorbic acid will be increased, but not the time at which the peak of the rise is reached. This was studied for subject D and the data are summarized in table 2. When the fasting level was 1.4 mg. per 100 ml. plasma, the ingestion of 50 mg. crystalline ascorbic acid caused an increase by 9:30 a.m. of from 0.33 to 0.42 mg. However, at a later date when the fasting level had been dropped to 0.8 mg. per 100 ml. of plasma the ingestion of 50 mg. ascorbic acid caused an increase of from 0.16 to 0.28 mg. per 100 ml. The fasting level was again lowered by going on a diet from which all fruits and vegetables were excluded; in 15 days the plasma content dropped from a level of 1.28 mg. to 0.48 mg. per 100 ml. At this level the ingestion of 50 mg. ascorbic acid failed to produce any significant change in plasma ascorbic acid on 3 successive days. Therefore, on each of the next 3 days 150 mg. crystalline ascorbic acid was ingested and a significant increase was obtained by 9:30 a.m. each day.

Influence of amount of ascorbic acid ingested

Subject E was followed through a series of determinations in which the fasting level was approximately the same but the amount ingested was varied as 0, 50, 100, 200 and 400 mg. crystalline ascorbic acid. Typical curves for this subject at each level of intake are shown in figure 1. With no intake of ascorbic acid there was no change in the ascorbic acid content of the blood plasma; a similar finding was reported by Taylor, Chase and Faulkner ('36). As the intake of ascorbic acid was increased, the plasma content of this substance increased more rapidly and reached a correspondingly greater maximum until

TABLE 2

Ascorbic acid content of blood plasma of one subject with different initial levels and intake.

DATE	SUPPLEMENT	MG. ASCORBIC ACID PER 100 ML. PLASMA AT GIVEN HOUR IN A.M.										MAXIMUM INCREASE
		8:00	8:15	8:30	9:00	9:30	10:00	10:30	11:00	11:30	12:00	
												mg.
Nov. 15 '39	50 mg.	1.39	—	—	1.43	1.66	1.81	1.70	1.68	1.66	1.45	0.42
Nov. 16	ascorbic	1.43	—	—	1.51	1.68	1.68	1.76	1.68	—	1.51	0.33
Nov. 17	acid [1]	1.41	—	—	1.45	1.64	1.68	1.76	1.68	1.60	1.51	0.35
Feb. 19 '40	50 mg.	0.92	0.88	0.92	1.10	1.10	1.20	1.04	0.99	0.94	0.90	0.28
Feb. 20	ascorbic	0.94	0.92	0.94	1.04	1.10	1.04	1.02	0.97	0.94	0.97	0.16
Feb. 21	acid	0.88	0.88	0.97	1.08	1.06	1.06	0.97	0.88	0.88	0.90	0.20
April 1 '40	No fruits or	1.28										
April 10	vegetables	0.72										
April 12		0.59										
April 15	50 mg.	0.48	0.46	0.44	0.44	0.48	0.44	0.44	0.44	0.44	0.40	—
April 16	ascorbic	0.35	0.38	0.35	0.38	0.44	0.35	0.35	0.35	0.35	0.35	—
April 17	acid	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	—
April 18	150 mg.	0.35	0.35	0.35	0.53	0.62	0.53	0.44	0.46	0.44	0.44	0.27
April 19	ascorbic	0.44	0.44	0.44	0.44	0.53	0.58	0.53	0.53	0.44	0.44	0.14
April 20	acid	0.44	0.44	0.44	0.44	0.55	0.53	0.48	0.44	0.44	0.46	0.11

[1] Supplement taken at 8:15.

with intakes of 200 and 400 mg. of ascorbic acid the plasma level failed to return to the original fasting level within 4 hours.

DISCUSSION

The data summarized in table 1 and figure 1 show the rapidity with which ascorbic acid is absorbed and appears in the blood stream; they also show that when the intake is 50 mg. ascorbic acid, that is, an amount which might normally be taken at breakfast in a serving of fruit or in a small glass of orange juice, the blood plasma returns to the original fasting level in 3 to 4 hours after the meal. Alt, Chinn and Farmer ('39) have similarly reported that for six normal subjects, with an intake of 75 to 100 mg. ascorbic acid as orange juice taken at breakfast, the plasma ascorbic acid returned to the original fasting level 3 hours after the meal. Even when the intake is high, as with amounts of 400 mg. ascorbic acid, the plasma concentration tends to return to the original fasting level. When it is not practicable to obtain blood samples prior to breakfast, samples may be taken in the morning if no fruit or other source of ascorbic acid has been consumed within 4 or 5 hours.

Some investigators have questioned the value of plasma ascorbic acid as a measure of nutritional status because the values fall much more rapidly in the plasma than in the white cells (Crandon, Lund and Dill, '40; Butler and Cushman, '40) and because plasma values of practically zero have been obtained without any obvious signs of scurvy. However, the data obtained in the present study indicate that the fasting plasma level tends to remain constant from day to day with a diet of approximately the same intake, and when vitamin C-containing foods are withdrawn from the diet (table 2) there is a decrease in the plasma ascorbic acid content. The ascorbic acid content of the plasma reflects the dietary intake of this vitamin and although low plasma concentration is apparently not an indication of subclinical scurvy, these findings are believed to be indicative of the value of plasma determinations

as a measure of the state of vitamin C nutrition particularly for group studies. Recently Bryan and co-workers ('41) have also offered evidence for the close relation between dietary intake and plasma concentration of ascorbic acid.

SUMMARY

Five subjects, studied for rate of increase in plasma ascorbic acid after ingestion of 50 mg. of this vitamin, showed close similarity in response for the different subjects and for the same subject at different times.

The ascorbic acid content of blood plasma began to rise within 30 to 60 minutes after ingestion of 50 mg. of the vitamin either in crystalline form or from fruits, and returned to the fasting level in 3 to 4 hours.

The maximum increase in plasma level of ascorbic acid was reached within 1½ hours after ingestion of 50 mg. of this substance in either the crystalline form, as orange juice, or as orange sections; in 2 hours when strawberries were the source of ascorbic acid; and in 2½ hours when cauliflower was eaten.

The curve for increase of plasma ascorbic acid was not influenced by fat or by ferrous or ferric salt taken at the same time as the ascorbic acid.

When the intake was raised to 400 mg. ascorbic acid there was a greater increase in plasma concentration and a longer time elapsed before returning to the original fasting level.

Ingestion of 50 mg. ascorbic acid did not cause an increase in its plasma concentration when the fasting level was 0.5 mg. or less per 100 ml. plasma.

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Conservation of Scholarly Journals

The American Library Association created this last year the Committee on Aid to Libraries in War Areas, headed by John R. Russell, the Librarian of the University of Rochester. The Committee is faced with numerous serious problems and hopes that American scholars and scientists will be of considerable aid in the solution of one of these problems.

One of the most difficult tasks in library reconstruction after the first World War was that of completing foreign institutional sets of American scholarly, scientific, and technical periodicals. The attempt to avoid a duplication of that situation is now the concern of the Committee.

Many sets of journals will be broken by the financial inability of the institutions to renew subscriptions. As far as possible they will be completed from a stock of periodicals being purchased by the Committee. Many more will have been broken through mail difficulties and loss of shipments, while still other sets will have disappeared in the destruction of libraries. The size of the eventual demand is impossible to estimate, but requests received by the Committee already give evidence that it will be enormous.

With an imminent paper shortage attempts are being made to collect old periodicals for pulp. Fearing this possible reduction in the already limited supply of scholarly and scientific journals, the Committee hopes to enlist the cooperation of subscribers to this journal in preventing the sacrifice of this type of material to the pulp demand. It is scarcely necessary to mention the appreciation of foreign institutions and scholars for this activity.

Questions concerning the project or concerning the value of particular periodicals to the project should be directed to Wayne M. Hartwell, Executive Assistant to the Committee on Aid to Libraries in War Areas, Rush Rhees Library, University of Rochester, Rochester, New York.